Functional Genomics Subject Notes

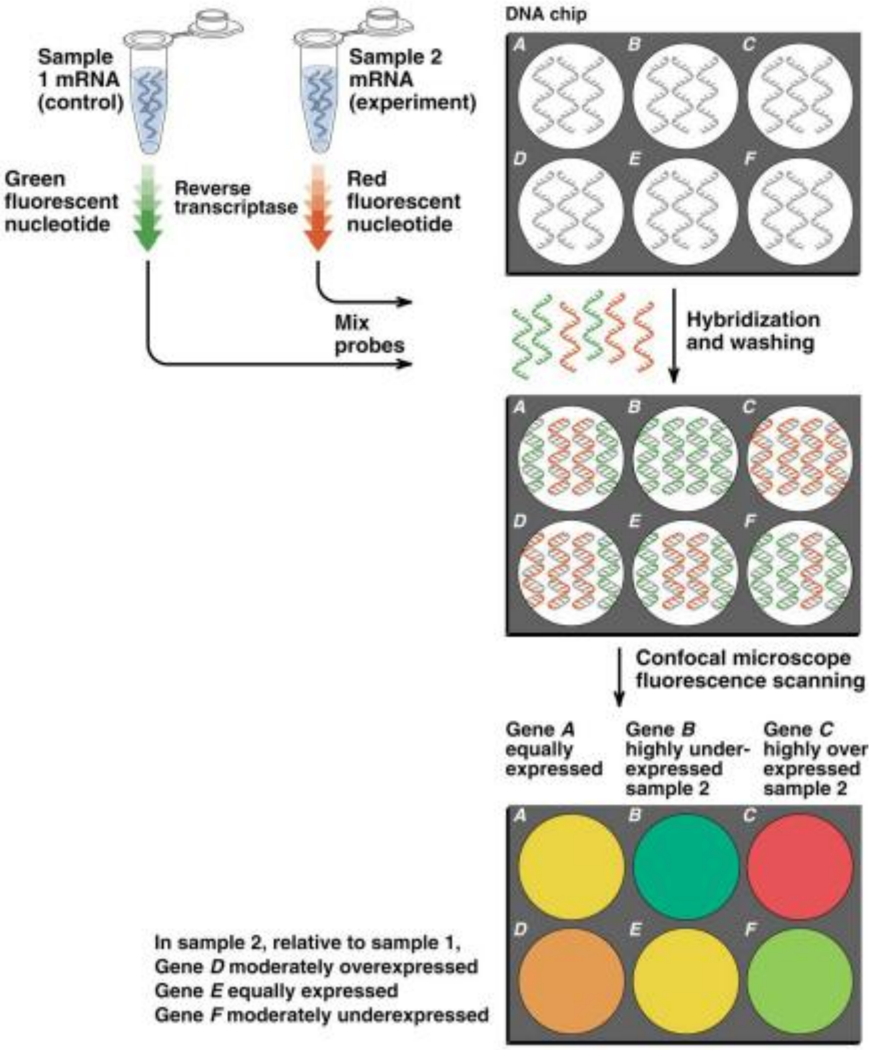
Introduction

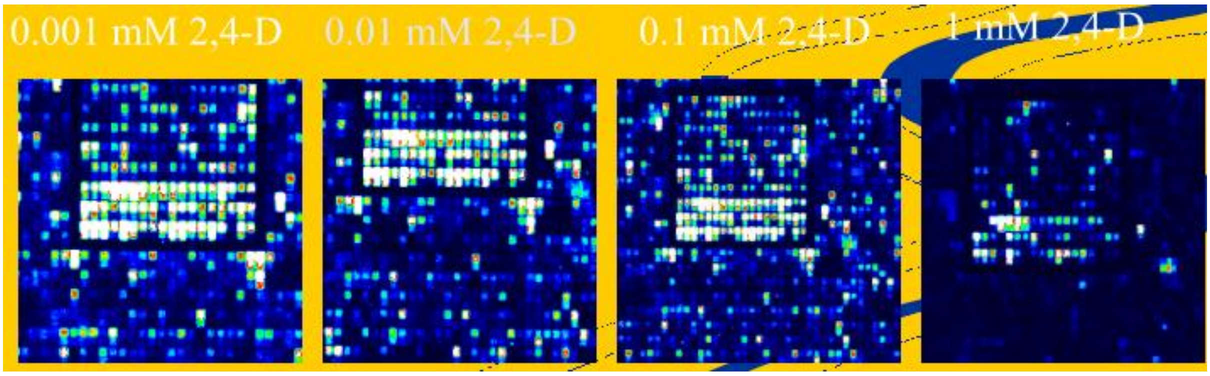
Structural vs functional genomics

- Structural genomics studies the physical nature of whole genomes, including the chromosome number, genome size, number of ORFs, linkage maps, etc
- Functional genomics studies the patterns of gene transcription and interaction as they relate to expression of the proteome
- Central dogma of genomics: genome -> transcriptome -> proteome -> phenotype
- The functional genomics of an organism depend upon the time of development, the region and type of tissue examined, and the physiological or pathological state of the organism

Microarrays

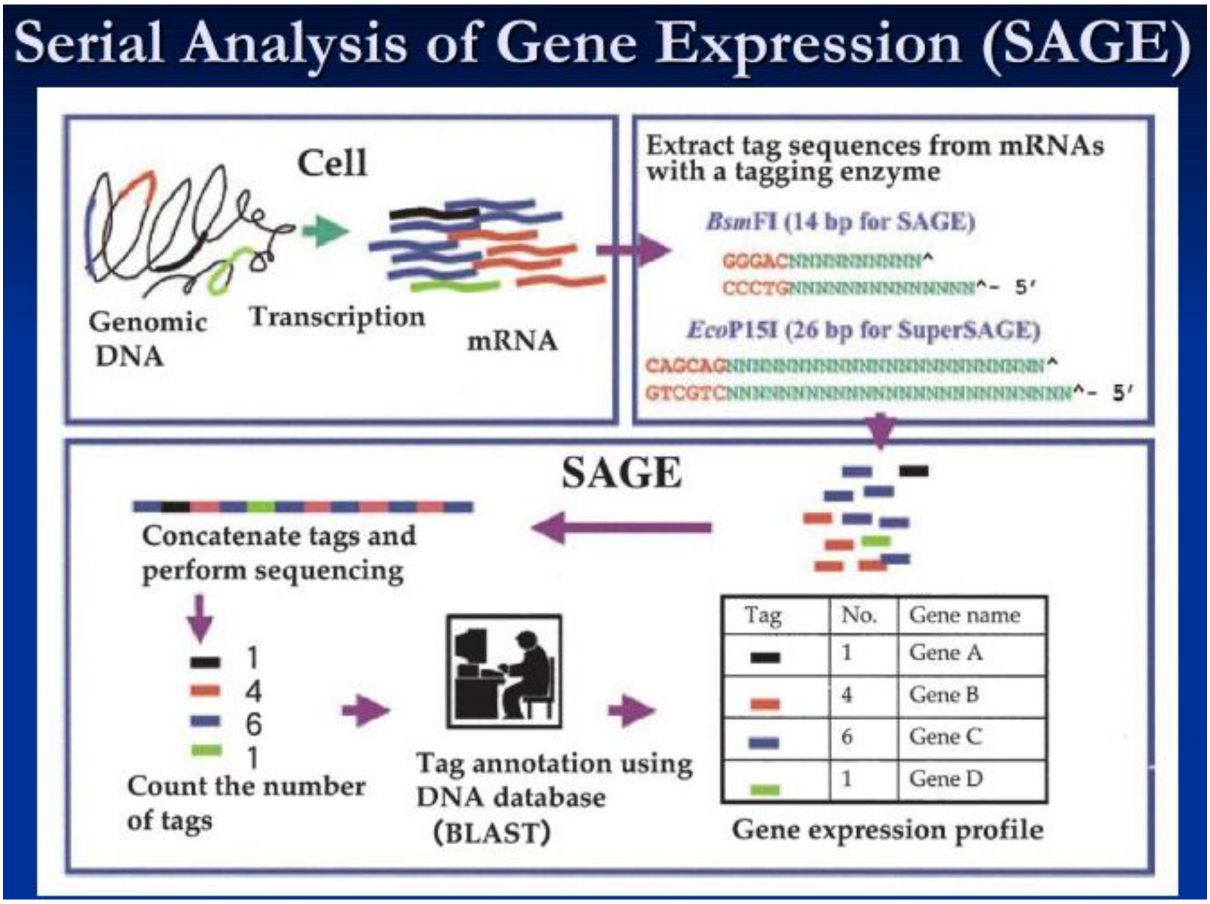
- DNA microarrays are used to study the expression of entire genomes. They involve PCR-generated cDNA products are arrayed using a robotic arm and competitively hybridised to Cy3 and Cy5 labelled RNA samples for expression analysis
- The results below show how addition of higher concentrationso f 2,4-D herbicide systematically reduce gene expression in Arabidopsis plants


Sample 1 mRNA (control)
Sample 2 mRNA (experiment)
Green fluorescent nucleotide
Reverse transcriptase
Red fluorescent nucleotide
Mix probes
DNA chip
A
B
C
D
E
F
Hybridization and washing
Confocal microscope fluorescence scanning
Gene A equally expressed
Gene B highly under-expressed sample 2
Gene C highly over expressed sample 2
In sample 2, relative to sample 1,
Gene D moderately overexpressed
Gene E equally expressed
Gene F moderately underexpressed



Serial analysis of gene expression

- Serial Analysis of Gene Expression (SAGE) is another technique which identifies mRNA transcripts by identifying small fragments of those transcripts cut by a Type III restriction enzyme



Serial Analysis of Gene Expression (SAGE)

Extract tag sequences from mRNAs with a tagging enzyme

*Bsm*FI (14 bp for SAGE)

GGGACNNNNNNNNNNN^
CCCTGNNNNNNNNNNNNNNN^- 5′

*Eco*P15I (26 bp for SuperSAGE)

CAGCAGNNNNNNNNNNNNNNNNNNNNNNNNNN^
GTCGTCNNNNNNNNNNNNNNNNNNNNNNNNNNNN^- 5′

SAGE

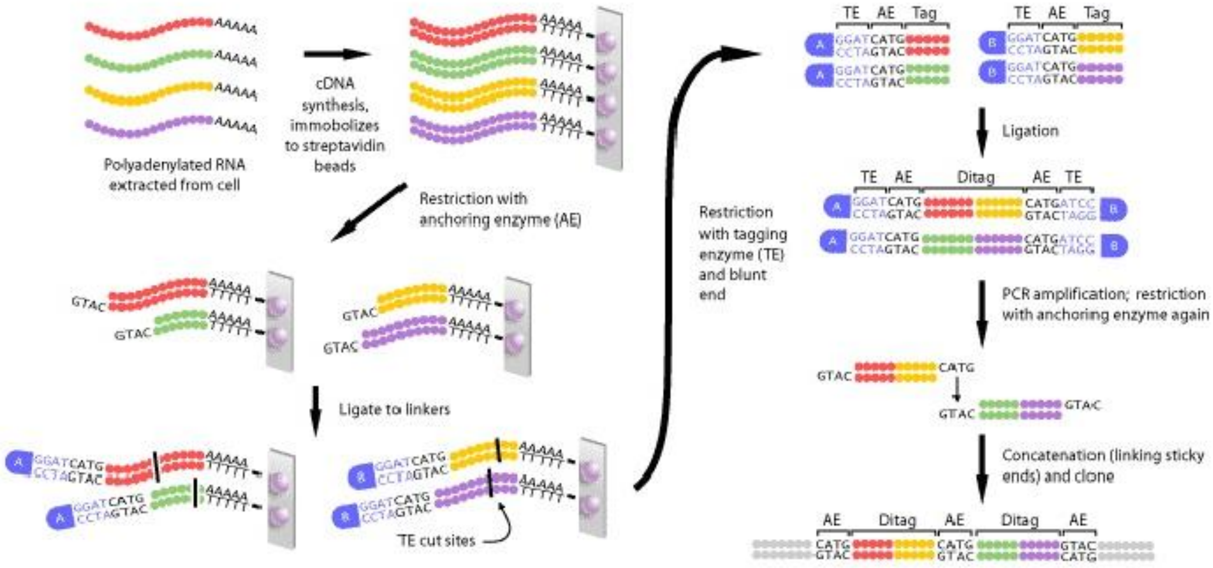
Concatenate tags and perform sequencing

Count the number of tags

Tag annotation using DNA database (BLAST)

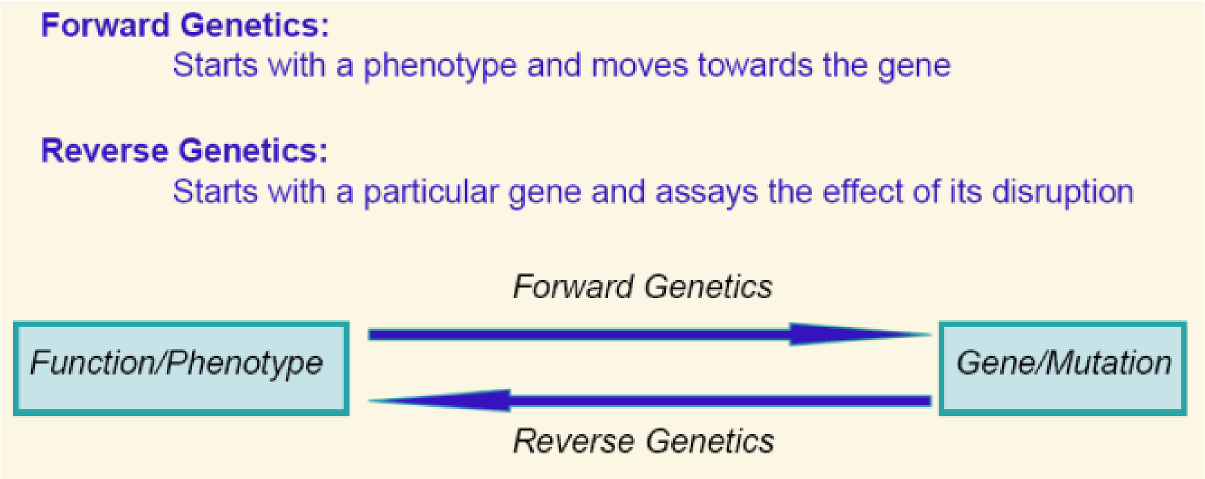
Tag	No.	Gene name
▬	1	Gene A
🟥	4	Gene B
🟦	6	Gene C
🟩	1	Gene D

Gene expression profile


Polyadenylated RNA extracted from cell
cDNA synthesis, immobolizes to streptavidin beads
Restriction with anchoring enzyme (AE)
GTAC
Ligate to linkers
TE cut sites
Restriction with tagging enzyme (TE) and blunt end
TE AE Tag
Ligation
TE AE Ditag AE TE
PCR amplification; restriction with anchoring enzyme again
CATG
GTAC
Concatenation (linking sticky ends) and clone
AE Ditag AE Ditag AE

Forward and reverse genetics

- Forward and reverse genetics are two complementary techniques for studying the relationship between phenotype and genotype
- Forward genetics typically proceeds by first introducing mutations into population using ionising radiation, chemicals (e.g. EMS), and then screening for a phenotype of interest. Genetic crosses are then used to map the locus of the phenotype to a particular gene location (e.g. F2 population), following by isolation and sequencing of the gene
- Reverse genetics starts with the known gene of interest and examines the effects of disrupting or modifying that gene (knockout)



Forward Genetics:
Starts with a phenotype and moves towards the gene

Reverse Genetics:
Starts with a particular gene and assays the effect of its disruption

Forward Genetics

Function/Phenotype

Gene/Mutation

Reverse Genetics

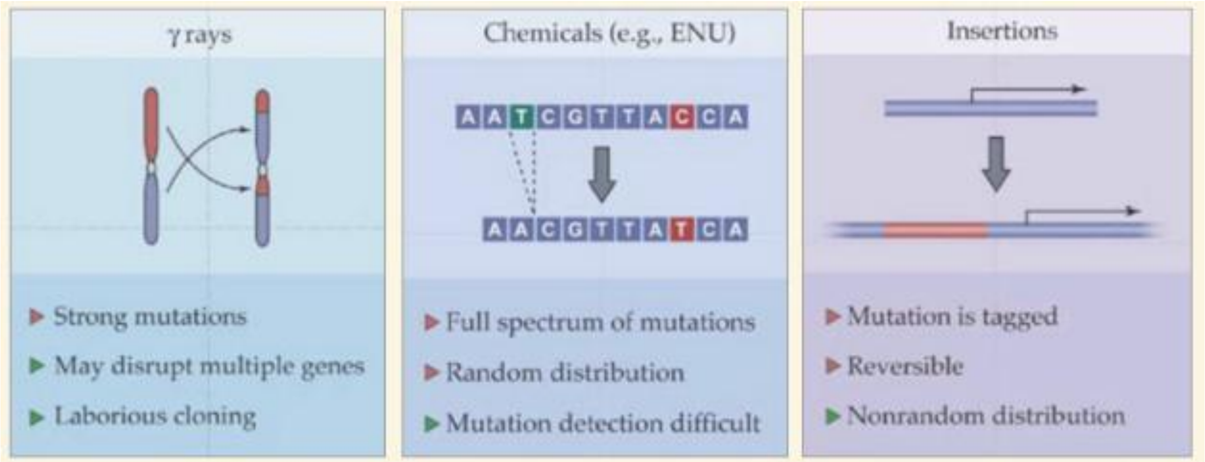
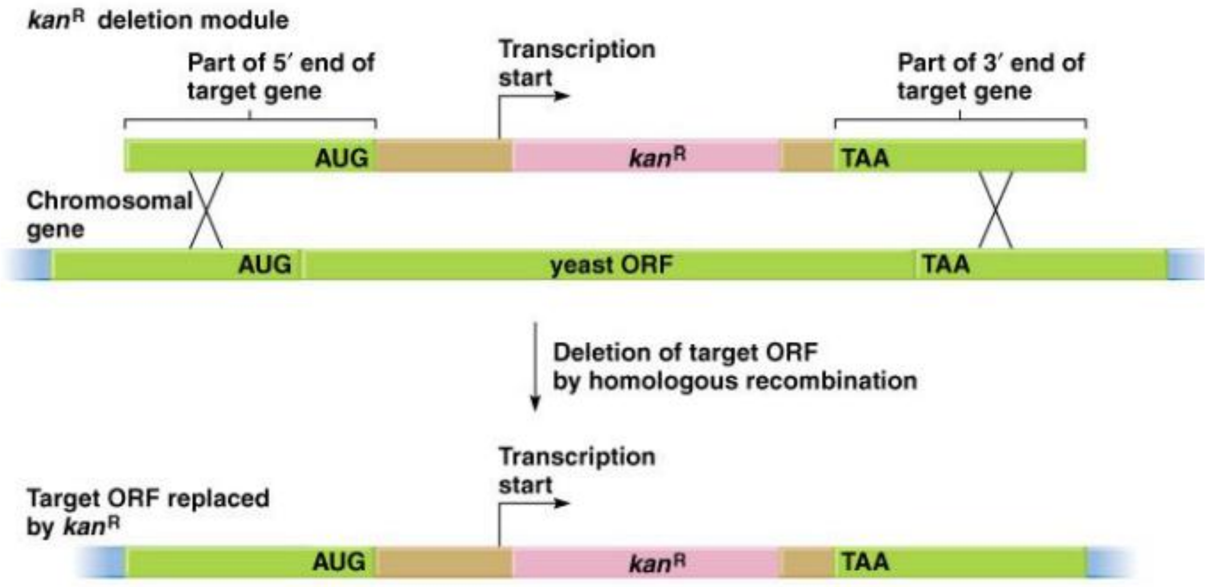


Fig. 9.15 Creating a gene knockout in yeast

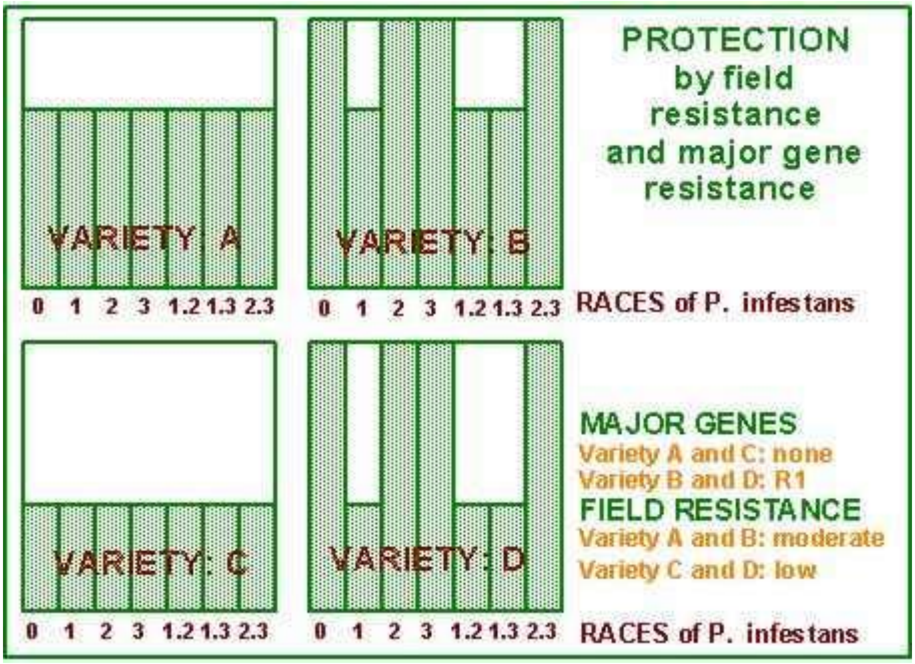

kanR deletion module
Part of 5′ end of target gene
Transcription start
Part of 3′ end of target gene
AUG
kanR
TAA
Chromosomal gene
AUG
yeast ORF
TAA
Deletion of target ORF by homologous recombination
Transcription start
Target ORF replaced by kanR
AUG
kanR
TAA

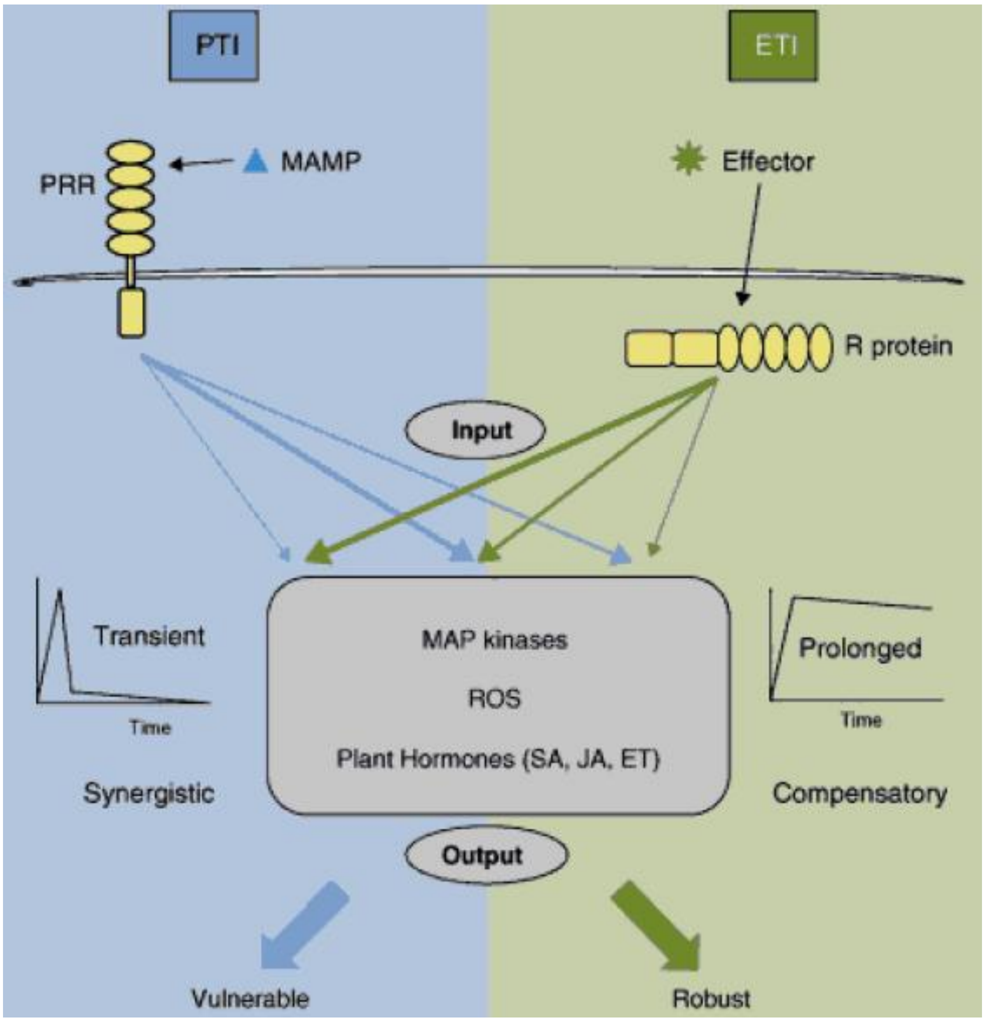
- Targeted Induced Local Lesions IN Genomes (TILLING) is a reverse genetics technique for knocking out specific genes of interest. The method combines a standard and efficient technique of mutagenesis using a chemical mutagen such as Ethyl methanesulfonate (EMS) with a sensitive DNA screening-technique that identifies single base mutations
- RNA interference is another method that can be used to silence the expression of particular genes

Plant Disease Resistance

Overview

- Vertical resistance is race/pathotype specific, while horizontal resistance is effective against all pathotypes (general resistance)
- The gene-for-gene hypothesis holds that "each resistance gene in the host plant is matched by a corresponding virulence gene in the pathogen"
- This can be described as a series of resistance gene 'locks' and pathogenicity 'keys'

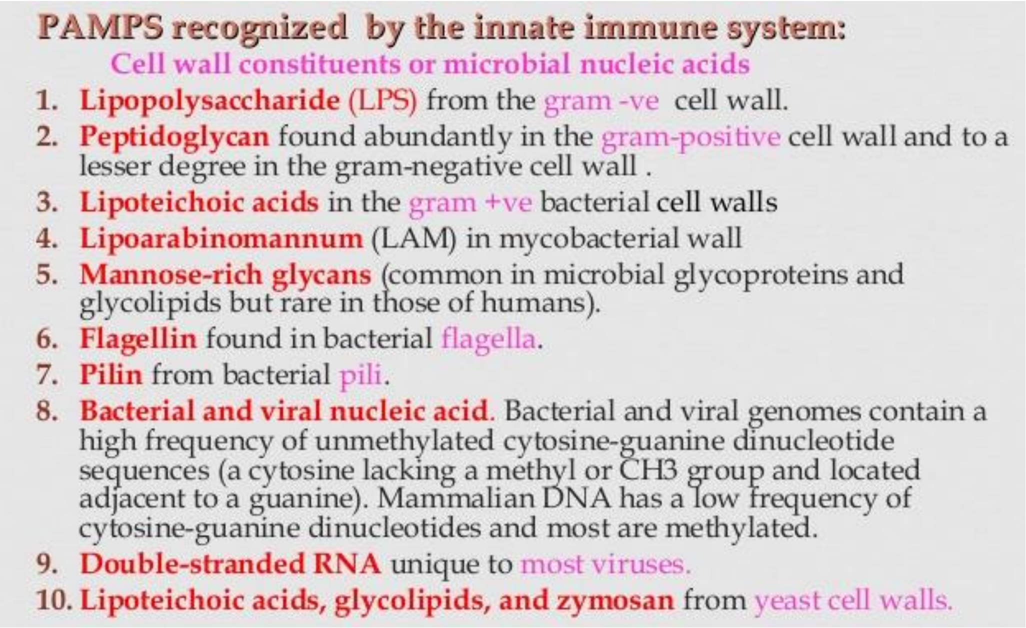




Plant innate immunity

- Pathogen associated molecular patterns (PAMPs) are molecules associated with potential pathogens that are recognised by proteins embedded in the plant cell membrane called pattern recognition receptors (PRRs)

- PAMPs (keys) mutate to try to bypass or avoid the PRRs (locks)
- Examples of PAMPs:
    - Bacterial flagellin
    - Lipopolysaccharides (LPS)
    - Peptidoglycans
    - Glucans and glycoproteins
    - Chitins



PAMPS recognized by the innate immune system:

Cell wall constituents or microbial nucleic acids

1. **Lipopolysaccharide** (LPS) from the gram -ve cell wall.
2. **Peptidoglycan** found abundantly in the gram-positive cell wall and to a lesser degree in the gram-negative cell wall .
3. **Lipoteichoic acids** in the gram +ve bacterial cell walls
4. **Lipoarabinomannum** (LAM) in mycobacterial wall
5. **Mannose-rich glycans** (common in microbial glycoproteins and glycolipids but rare in those of humans).
6. **Flagellin** found in bacterial flagella.
7. **Pilin** from bacterial pili.
8. **Bacterial and viral nucleic acid**. Bacterial and viral genomes contain a high frequency of unmethylated cytosine-guanine dinucleotide sequences (a cytosine lacking a methyl or CH3 group and located adjacent to a guanine). Mammalian DNA has a low frequency of cytosine-guanine dinucleotides and most are methylated.
9. **Double-stranded RNA** unique to most viruses.
10. **Lipoteichoic acids, glycolipids, and zymosan** from yeast cell walls.

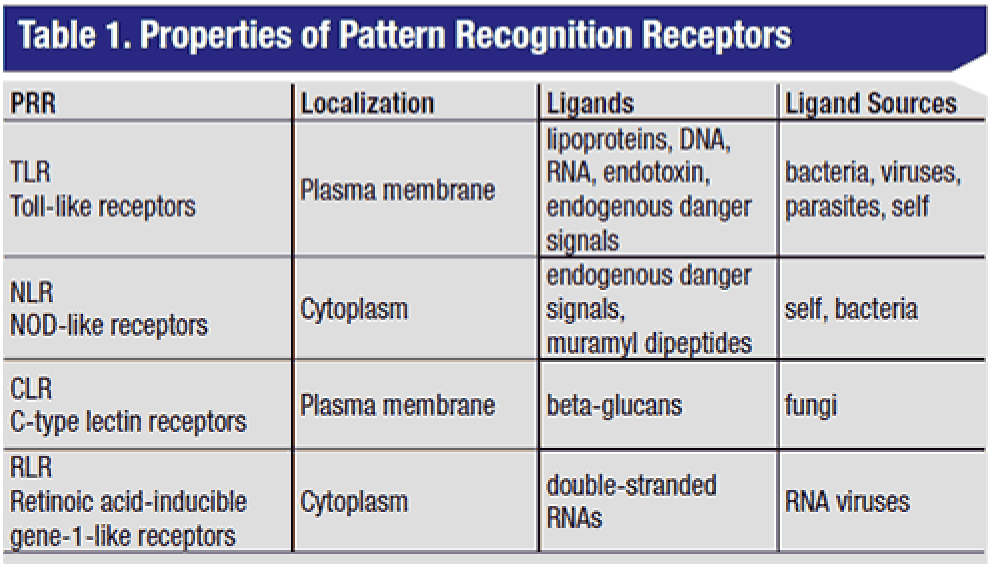
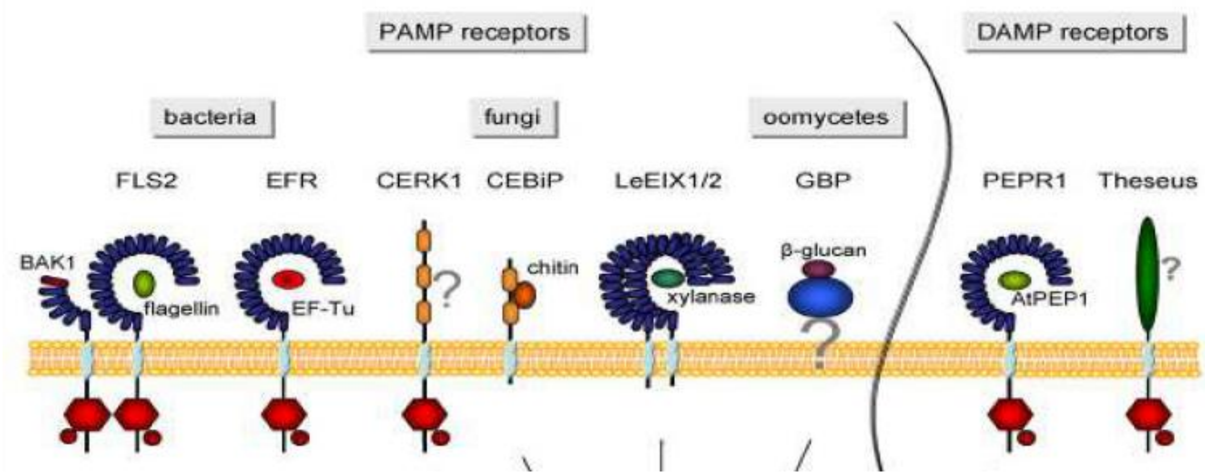


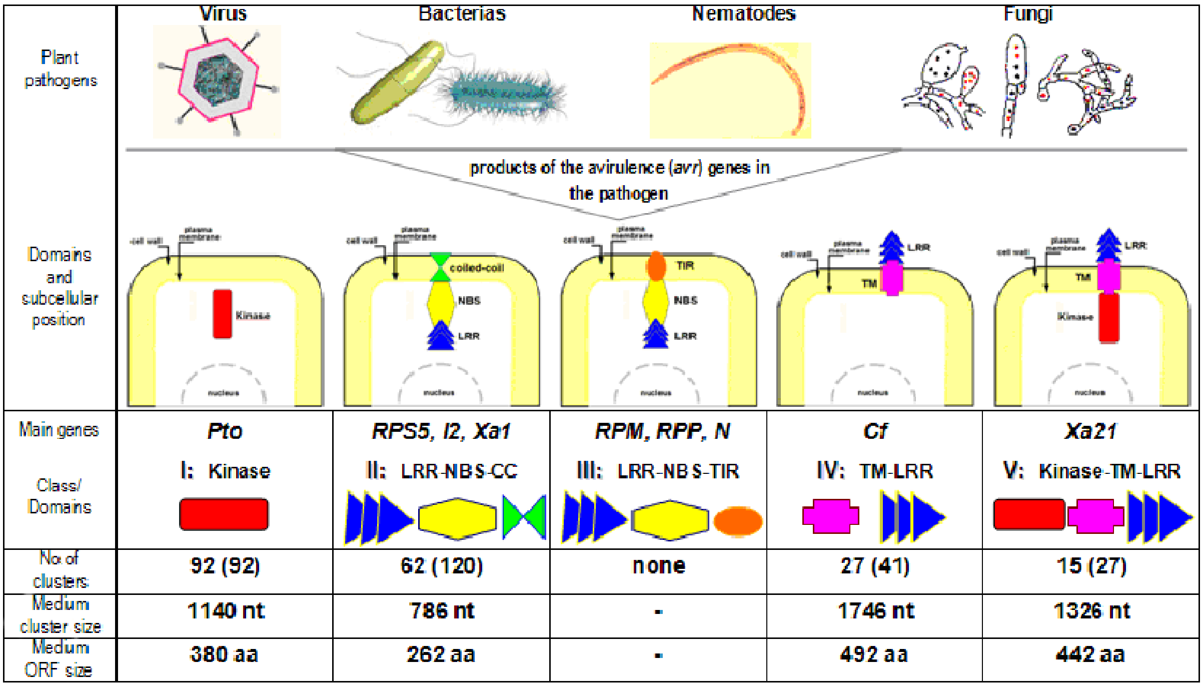
Table 1. Properties of Pattern Recognition Receptors

PRR	Localization	Ligands	Ligand Sources
TLR Toll-like receptors	Plasma membrane	lipoproteins, DNA, RNA, endotoxin, endogenous danger signals	bacteria, viruses, parasites, self
NLR NOD-like receptors	Cytoplasm	endogenous danger signals, muramyl dipeptides	self, bacteria
CLR C-type lectin receptors	Plasma membrane	beta-glucans	fungi
RLR Retinoic acid-inducible gene-1-like receptors	Cytoplasm	double-stranded RNAs	RNA viruses

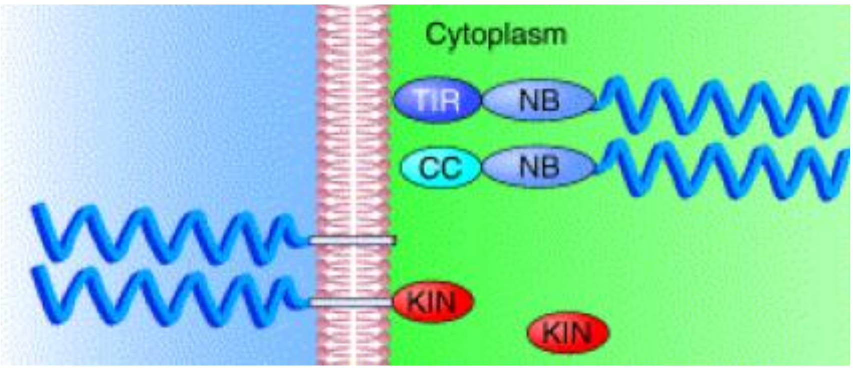


Effecter-triggered immunity

- Effecter-triggered immunity (ETI) is a form of acquired immunity mediated by R genes
- There are five different classes of R genes, all but the first of which are protein receptors
- Class 1: detoxifying enzymes (e.g. Hm1 gene)
- Class 2: intracellular serine/threonine kinases that function in signal transduction (e.g. Pto gene)
- Class 3a: intracellular proteins with Leucine Rich Repeats (LRRs) involved in protein-protein interactions, a Nucleotide Binding Site (NBS), and a coiled-coiled (CC) leucine zipper region (for gene regulation)
- Class 3b: identical to 3a but lacks a leucine zipper domain, and instead has a cytoplastic signalling domain called a Toll innate immunity receptor (TIR) (e.g. tobacco N gene)
- Class 4: contain extracellular leucine rich repeats and a transmembrane domain (e.g. Cf9 gene)
- Class 5: similar to class 4 but contains an additional intracellular serine/threonine kinase domain. These proteins can both recognise a pathogen gene product, and activate signal transduction (e.g. Xa21 gene)

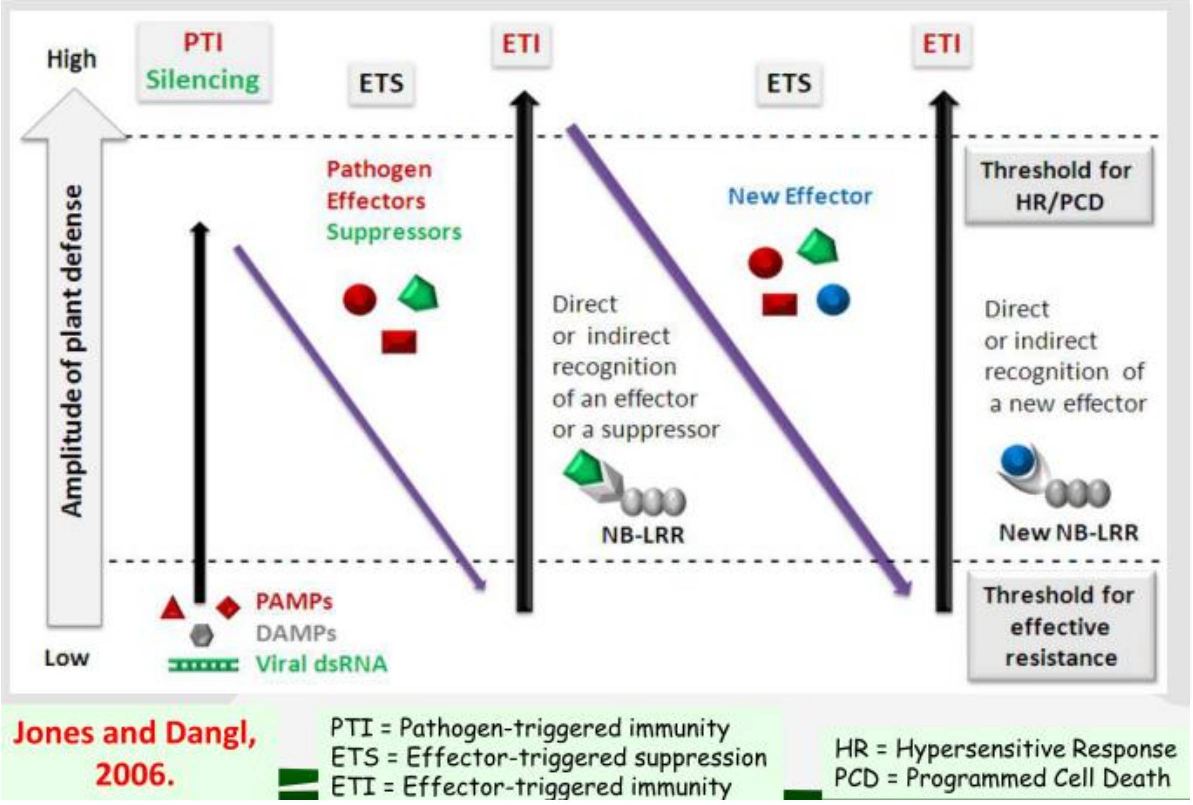

Plant pathogens
Virus
Bacterias
Nematodes
Fungi
products of the avirulence (avr) genes in the pathogen
Domains and subcellular position
cell wall
plasma membrane
Kinase
coiled-coil
NBS
LRR
TIR
TM
nucleus

Main genes	*Pto*	*RPS5, I2, Xa1*	*RPM, RPP, N*	*Cf*	*Xa21*
Class/ Domains	I: Kinase	II: LRR-NBS-CC	III: LRR-NBS-TIR	IV: TM-LRR	V: Kinase-TM-LRR
No of clusters	92 (92)	62 (120)	none	27 (41)	15 (27)
Medium cluster size	1140 nt	786 nt	-	1746 nt	1326 nt
Medium ORF size	380 aa	262 aa	-	492 aa	442 aa



The zig-zag model

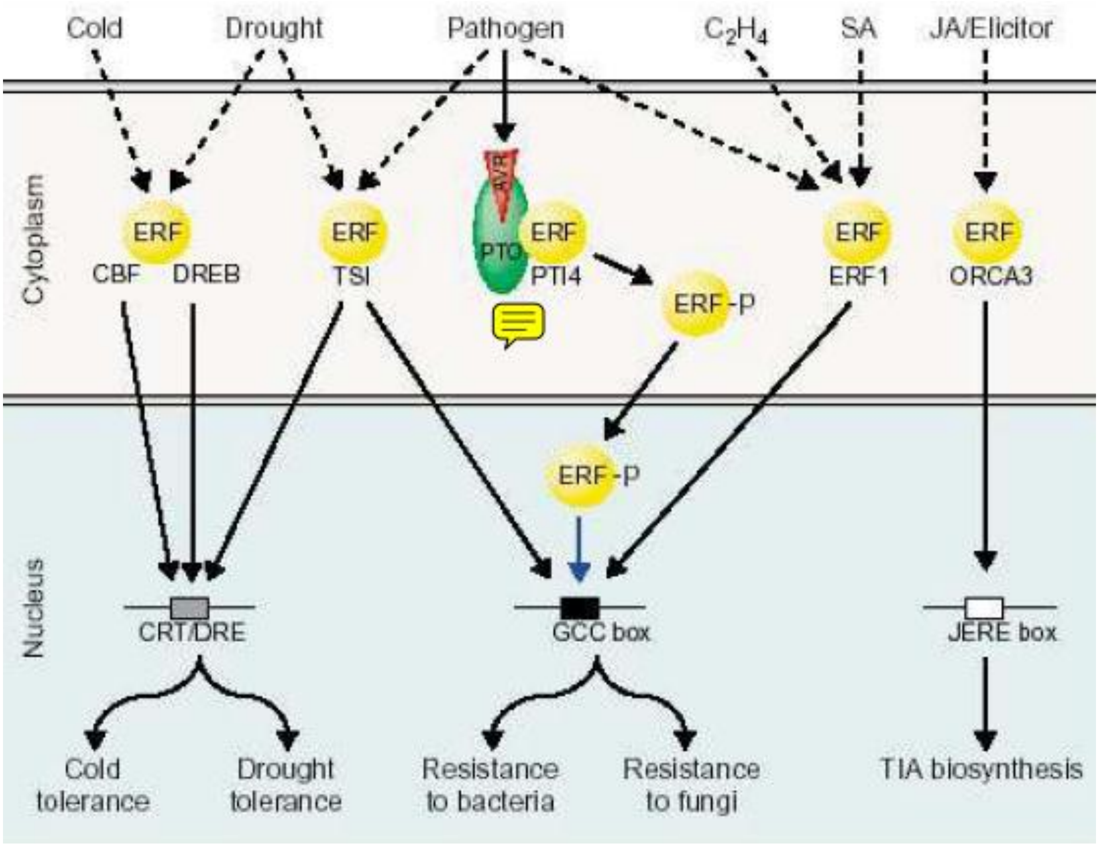
- In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]
- In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI)
- In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS)
- In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR)
- In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI

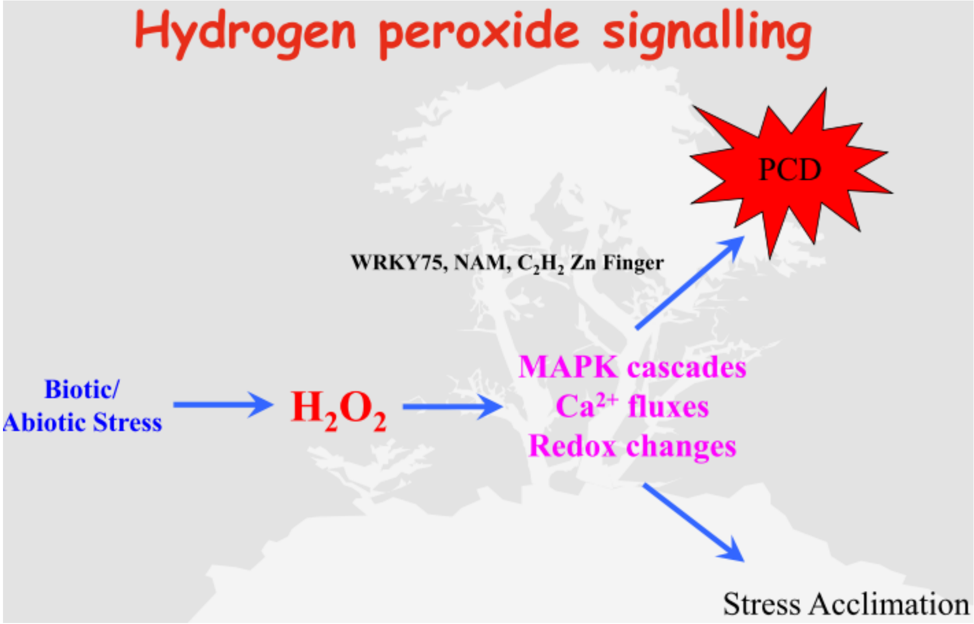


High
PTI
Silencing
ETS
ETI
ETS
ETI
Amplitude of plant defense
Pathogen
Effectors
Suppressors
New Effector
Threshold for HR/PCD
Direct or indirect recognition of an effector or a suppressor
Direct or indirect recognition of a new effector
NB-LRR
New NB-LRR
Threshold for effective resistance
Low
PAMPs
DAMPs
Viral dsRNA
Jones and Dangl, 2006.
PTI = Pathogen-triggered immunity
ETS = Effector-triggered suppression
ETI = Effector-triggered immunity
HR = Hypersensitive Response
PCD = Programmed Cell Death

Signalling genes

- Signalling genes are essential for transmitting the signal concerning the detection of the pathogen
- Ethylene-Responsive Element-Binding Proteins are transcription factors which respond to ethylene and are responsible for mediating plant adaptations to cold, drought, etc
- The salicylic/jasmonic acid pathways are involved in wounding response and responding to infections
- Hydrogen peroxide plays an important role in signalling a variety of biotic and abiotic stresses, triggering either adaptation or programmed cell death





Defensive responses

- Some types of defences attack the pathogen directly, for example by inhibiting spore germination or degrading the cell walls
  - Chitinase to attack cell wall
  - Protesase inhibitors
  - Phytoalexin production pathway
  - Pathogenesis-Related (PR) Proteins (antifungal, antimicrobial)
- Other responses attempt to contain the pathogen, for example by lignin deposition
- A third type of response focuses on minimising the potential damage to cells by toxins and enzymes

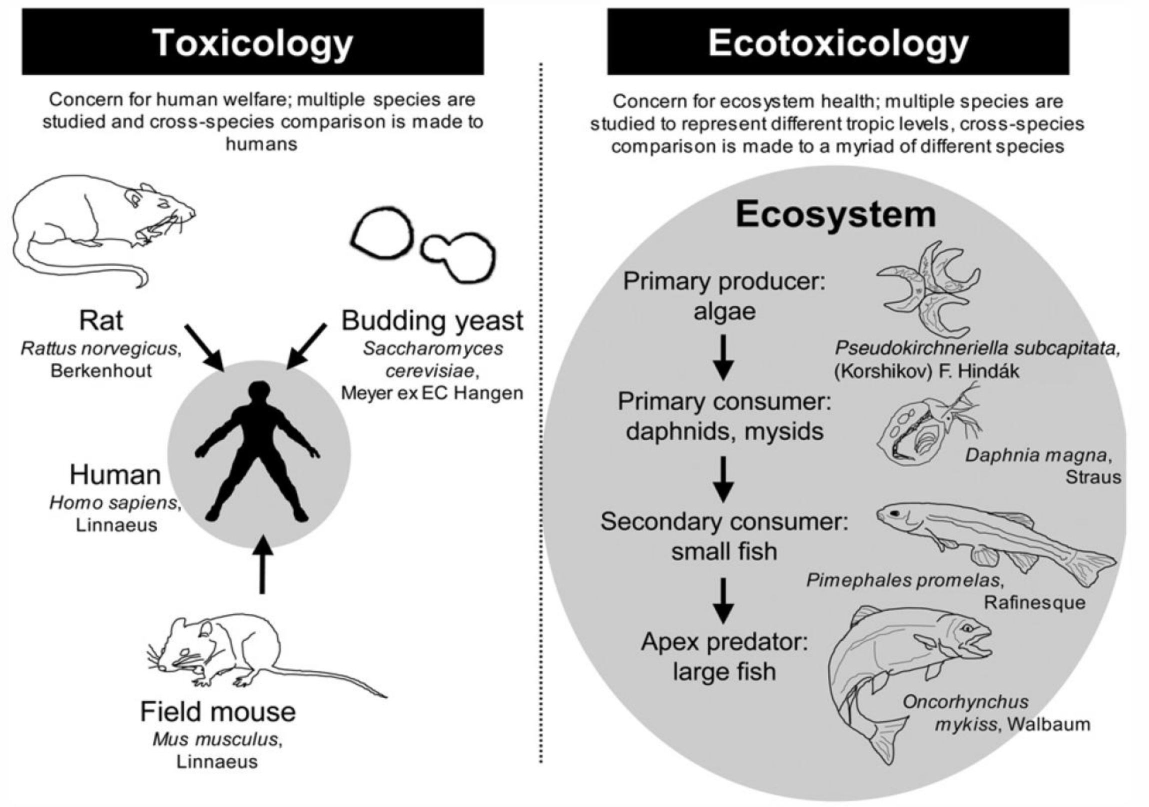
Chickpea case study

- Ascochyta Blight is a fungal pathogen affecting Australian chickpea crops
- The “Pulse Chip” is a microarray developed for chickpeas with the aim of looking for genes associated with disease resistance
- Gene expression studies were performed in ascochyta-blight resistant vs susceptible chickpea varieties
- Different genes show differing response patterns over time in susceptible and resistant varieties

Ecotoxicogenomics

Overview and terminology

- Ecotoxicology is the branch of toxicology concerned with the study of toxic effects caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial
- Lowest-observed-adverse-effect level (LOAEL): the lowest concentration of a substance found by experimentation which causes an adverse alteration of morphology, function, capacity, growth, development or life span of a target organism distinguished from normal organisms of the same species under defined conditions of exposure
- When the highest level of a substance is used and there is no observable effect, then: NOAEL
- No Observed Transcriptional Effect Level (NOTEL): the highest dose of chemical (environ. pollutant) which results in no significant changes in gene expression


Toxicology
Concern for human welfare; multiple species are studied and cross-species comparison is made to humans
Rat
Rattus norvegicus, Berkenhout
Budding yeast
Saccharomyces cerevisiae, Meyer ex EC Hangen
Human
Homo sapiens, Linnaeus
Field mouse
Mus musculus, Linnaeus
Ecotoxicology
Concern for ecosystem health; multiple species are studied to represent different tropic levels, cross-species comparison is made to a myriad of different species
Ecosystem
Primary producer: algae
Pseudokirchneriella subcapitata, (Korshikov) F. Hindák
Primary consumer: daphnids, mysids
Daphnia magna, Straus
Secondary consumer: small fish
Pimephales promelas, Rafinesque
Apex predator: large fish
Oncorhynchus mykiss, Walbaum

Features of ecotoxicology

- Often impossible to study multiple species in an ecosystem, so one or several Indicator Species (Bioindicators) are employed
- Common indicator species include:
  - *Daphnia magna* (water fleas)
  - *Danio rerio* (Zebrafish)
  - *Melanotaenia fluviatilis* (Murray River rainbowfish)
- Indicator species may vary from ecosystem to ecosystem, and may include plants

Common pollutants

- Heavy metal: mercury, cadmium, copper
- Pesticides: Chlorpyrifos, Diazinon
- Endocrine Disrupters: Oestrogen mimics
- Nanomaterials
- Environmental pollutants may induce gene-expression changes in indicator species (traditional focus is on morphology)
- These genomic changes may be indicative of the phase of the stress response that the species is undergoing
- Each phase of the stress response may be typified by characteristic patterns of gene expression

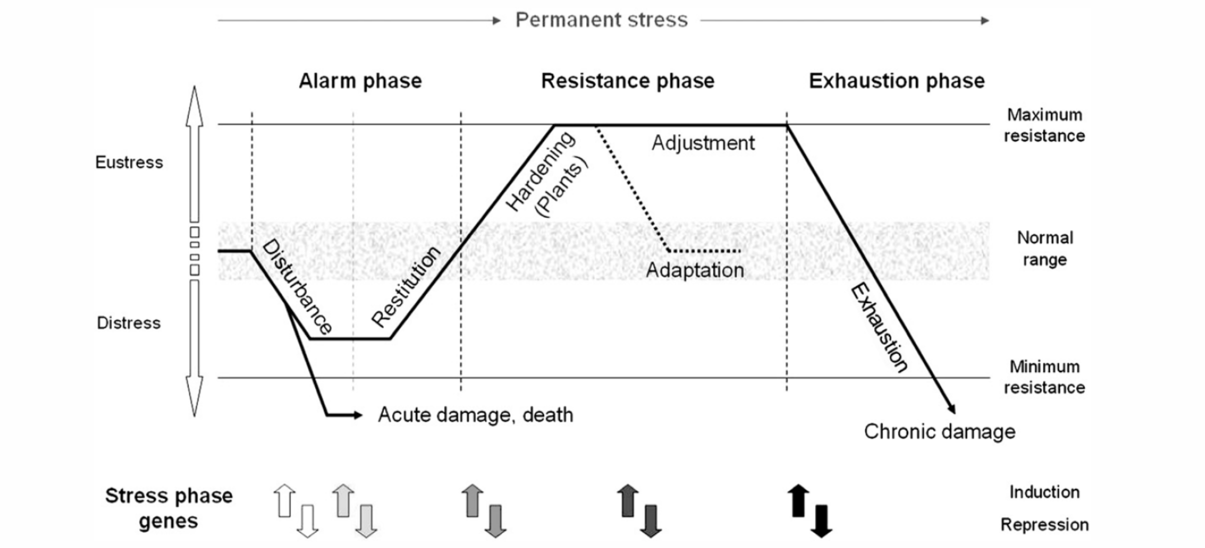
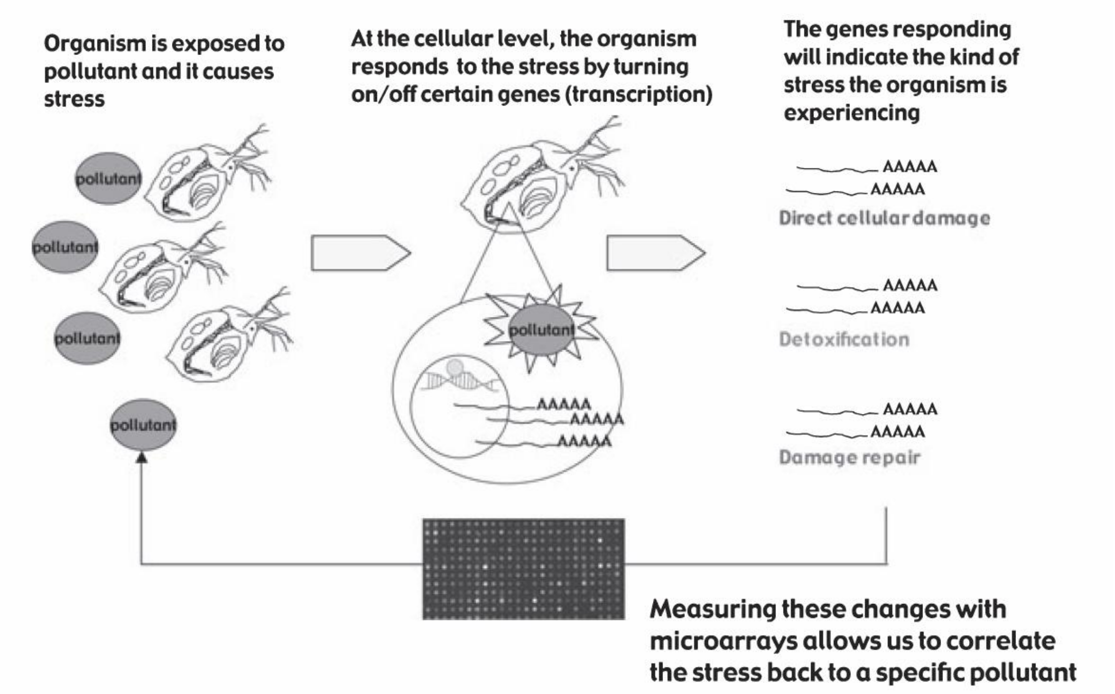


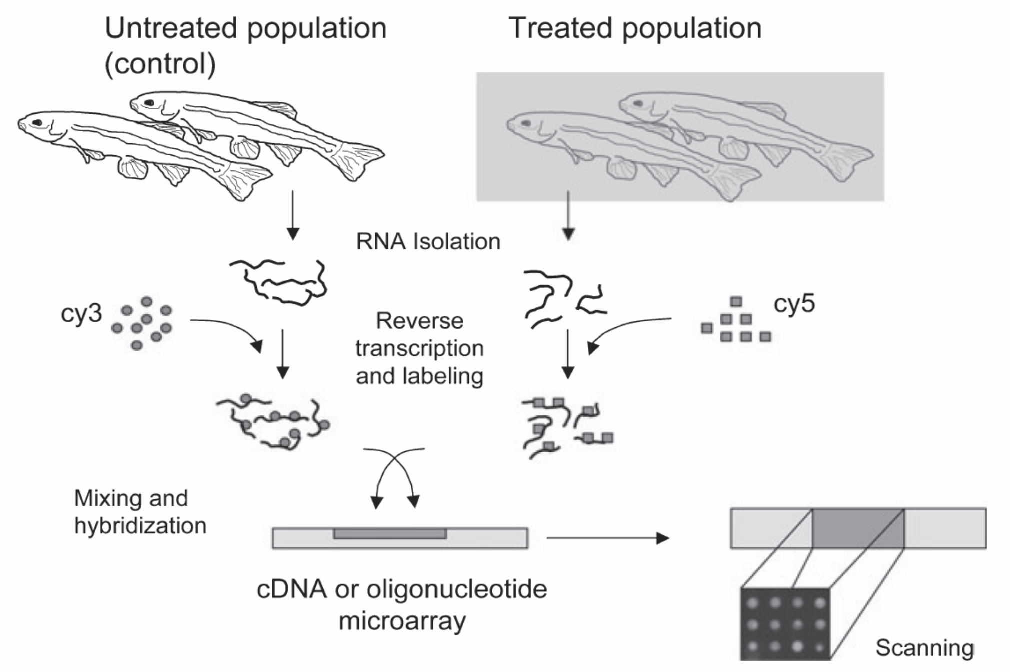
Fig. 1 – Stress phase model based on Selye (1936) and amended by several authors. Shades of grey of arrows represent different genes specifically expressed during the individual stress phases. Note, the gene profiles in the various stress phases are unique, even when exposed to the same stressor at a different intensity (see Section 2).

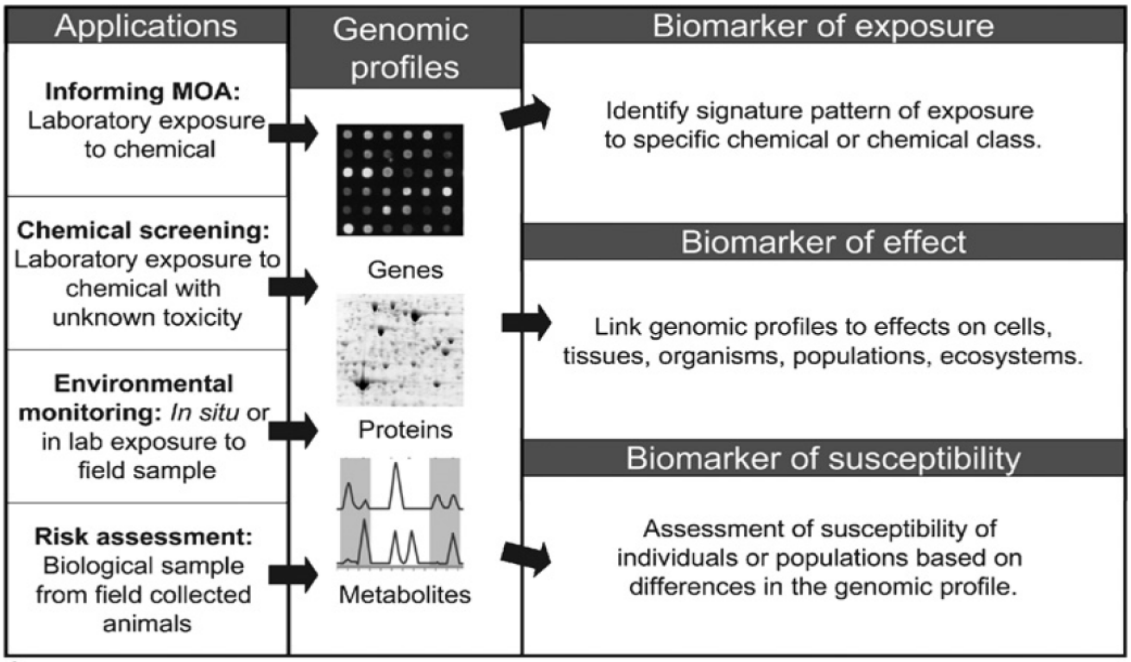
Genomics tools

- cDNA microarrays
- qRT-PCR
- SAGE
- Suppression subtractive hybridisation



Organism is exposed to pollutant and it causes stress
pollutant
pollutant
pollutant
pollutant
At the cellular level, the organism responds to the stress by turning on/off certain genes (transcription)
pollutant
AAAAA
AAAAA
AAAAA
The genes responding will indicate the kind of stress the organism is experiencing
AAAAA
AAAAA
Direct cellular damage
AAAAA
AAAAA
Detoxification
AAAAA
AAAAA
Damage repair
Measuring these changes with microarrays allows us to correlate the stress back to a specific pollutant


Untreated population (control)
Treated population
RNA Isolation
cy3
cy5
Reverse transcription and labeling
Mixing and hybridization
cDNA or oligonucleotide microarray
Scanning



Informing MOA: Laboratory exposure to chemical

Chemical screening: Laboratory exposure to chemical with unknown toxicity

Environmental monitoring: *In situ* or in lab exposure to field sample

Risk assessment: Biological sample from field collected animals

Identify signature pattern of exposure to specific chemical or chemical class.

Link genomic profiles to effects on cells, tissues, organisms, populations, ecosystems.

Assessment of susceptibility of individuals or populations based on differences in the genomic profile.

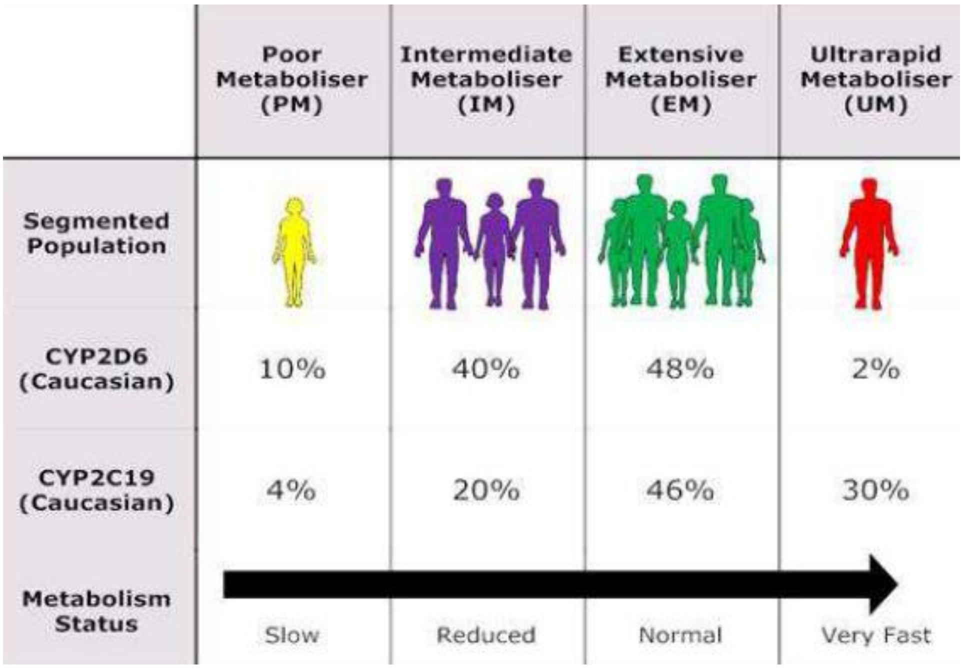
Advantages of genomics methods

- Sensitivity and early: phenotypic change may not manifest for days, and often it is possible to detect gene expression at toxicant concentrations too low to cause phenotypic change
- Specificity: Traditional approaches provide information of the method of action of a class of compounds, while whole-genome analysis can reveal differences in the MOA of closely-related compounds, because we can tell which specific genes are active, not just the overall phenotype
- More informative: traditional approaches only measure distress (e.g. growth rate changes), death, or reduced reproduction, whereas large transcriptomic data set from array studies provides much more information about expression levels and gene interactions
- Cross-species comparisons: gene sequence homology and common biochemical pathways between different species allow the extrapolation of experimental results to other species

Pharmacogenomics

Overview

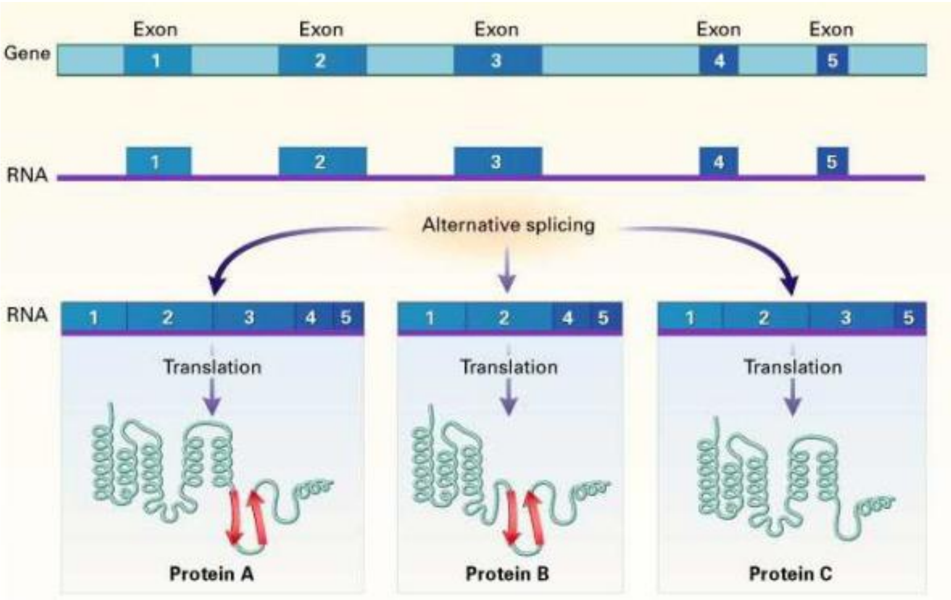
- Pharmacogenetics is the study of allelic variation in human populations that affect drug metabolism
- The field has traditionally studied single-gene traits with high penetrance (degree of connection between genotype and associated phenotype), e.g. genes encoding drug metabolising enzymes, drug transporters, and disease-modifying genes
- It has been defined as the use of genomics to get “the right dose of the right drug to the right patient at the right time”
- Fast, or slow metabolism of drugs affect retention of “parent” drug and the accumulation of the “metabolised” drug products
- Fast metabolism is good when the active drug is the metabolised product, but bad when the metabolised products are toxic

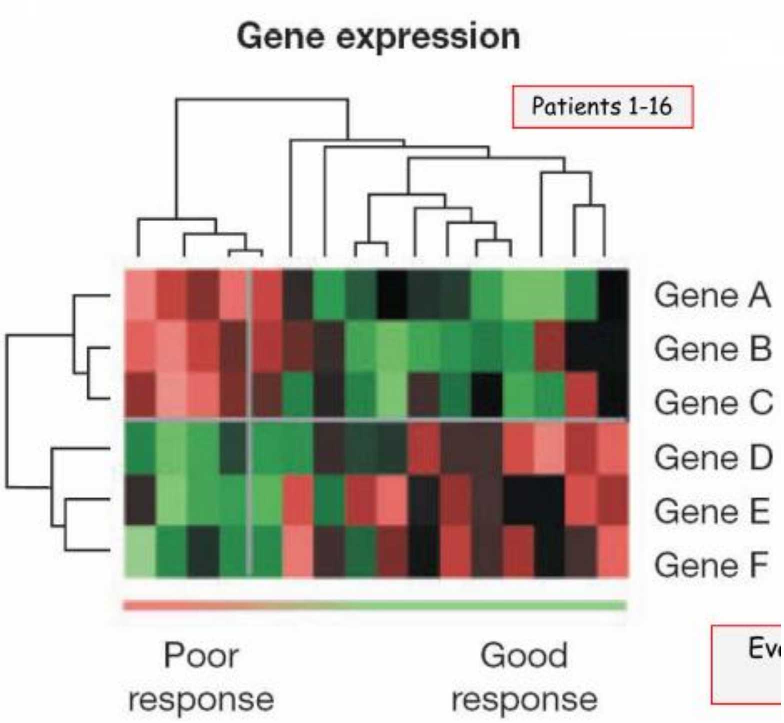


	Poor Metaboliser (PM)	Intermediate Metaboliser (IM)	Extensive Metaboliser (EM)	Ultrarapid Metaboliser (UM)
Segmented Population				
CYP2D6 (Caucasian)	10%	40%	48%	2%
CYP2C19 (Caucasian)	4%	20%	46%	30%
Metabolism Status	Slow	Reduced	Normal	Very Fast

Causes of pharmacological diversity

- Alternative splicing of human genes
- Presence or absence of drug transporters
- Allelic variation in drug targets and receptors
- Though some simple cases exist, most drug interactions are polygenetically controlled, thereby requiring genome-wide approaches (functional genomics and proteomics)





Abiotic Stress Resistance

Major abiotic stresses

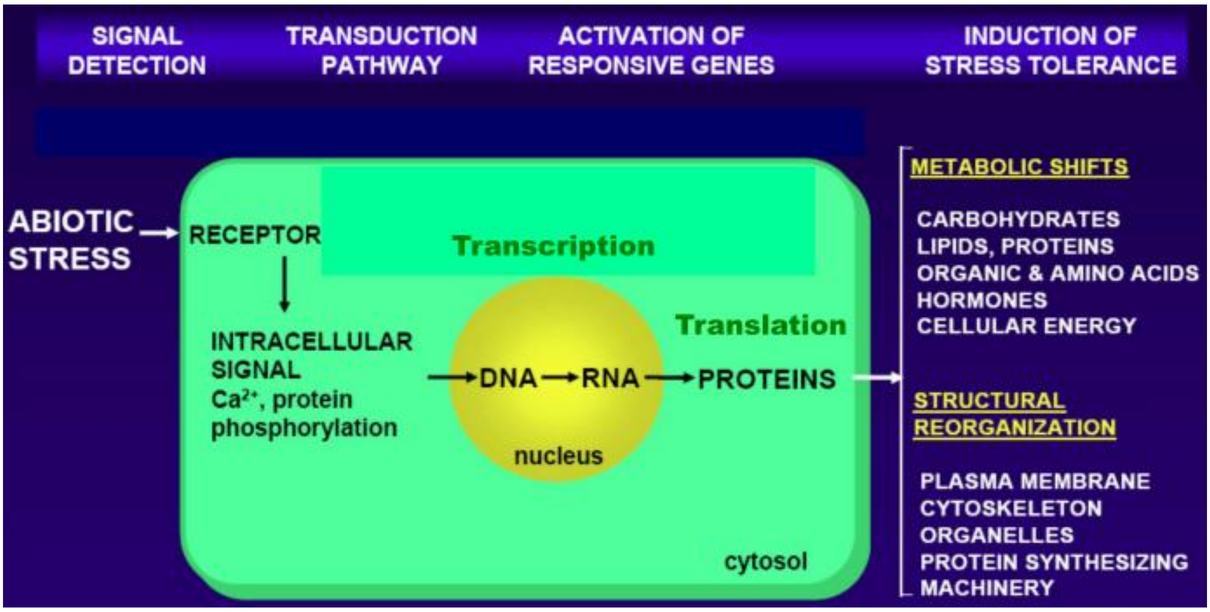
- Drought stress: water stress due to lack or insufficient rainfall or inadequate water supply
- Cold stress: temperature drops below the optimum, limiting growth and productivity
- Salinity stress: stress caused due to high concentration of soluble salts in the soil

Modes of stress resistance

- Escape (passive): ability of a plant to complete its life cycle before serious stress develops
- Avoidance (passive): ability of a plant to maintain growth in stressed environment using morphological features
- Tolerance (active) : ability of plant cells to produce metabolites/proteins to cope with stress conditions

Cellular events of tolerance

- The advent of abiotic stress is first sensed by receptor molecules usually attached to the cell membrane
- These receptor molecules generate a signal that is passed on to primary messengers (e.g. Ca 2+ dependent protein kinases)
- The primary sensors either result in direct activation of gene expression or relay the signal to secondary messengers that mediate specific gene expression by modifying the signal (e.g. phosphorylation of signalling molecules)
- The gene expression results in gene activation to produce messenger RNAs which code for specific proteins that effect stress tolerance either by themselves or by helping to synthesize specific metabolites (carbohydrates and lipids) that effect tolerance
- The proteins or metabolites effect stress tolerance by structural modification of plasma membrane, cytoskeleton, cell organelles or protein synthesizing machinery

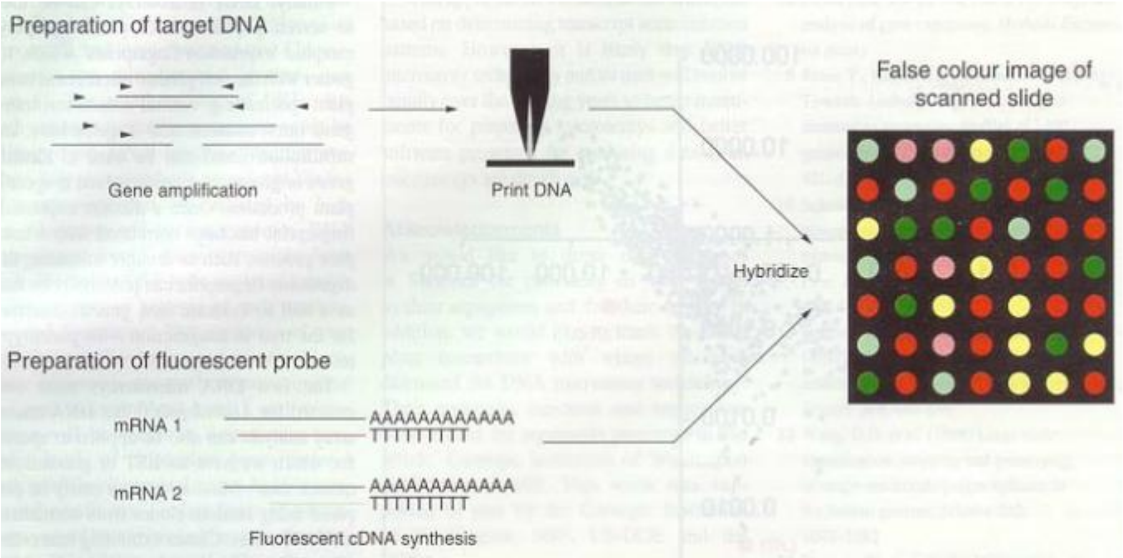


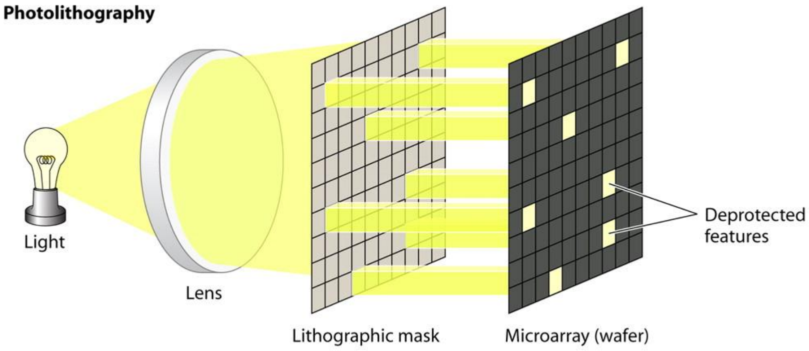
Using functional genomics to improve crop yield

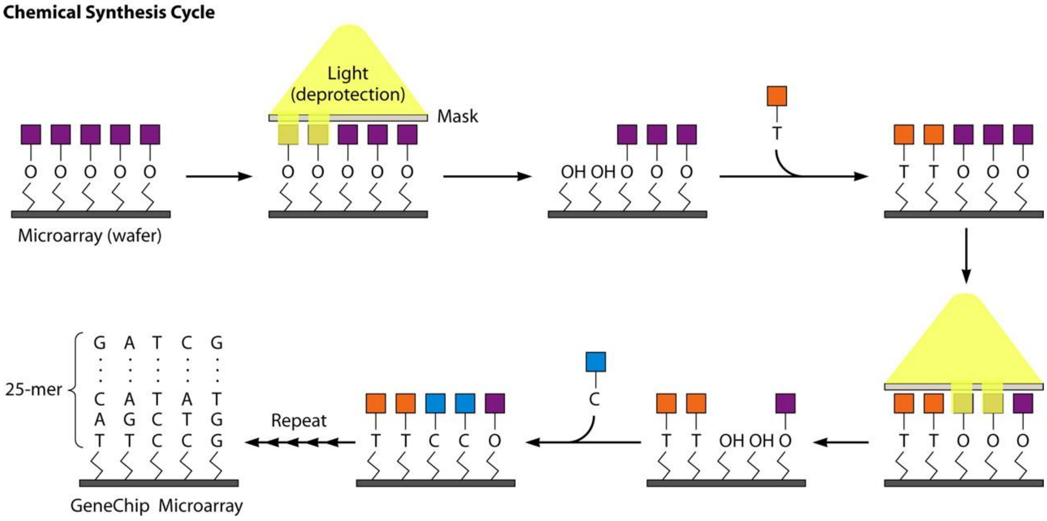
- The first step in the functional genomics approach is to generate genomic sequence information by sequencing coding and non-coding regions of DNA
- Using this sequence information, a global expression analysis is performed to compare genes (transcriptome studies) or proteins (proteomic studies) or metabolites (metabolomic studies) of stress tolerant and susceptible plants
- Transcriptomic studies are performed using techniques like RNA seq, microarrays & SAGE, while proteomic studies are performed using techniques like 2DGE & MALDI-TOF
- The genes or proteins thought to confer stress tolerance are validated using overexpressing transgenics, knockouts, repression using antisense RNAs or QTL mapping
- Validated genes are then tested in crop enhancement programs through molecular breeding or transgenics
- Successful multi-location trials of selected lines results in development of a variety tolerant to particular stress

Types of microarrays

- Spotted arrays: genes to be assayed are obtained as a set of plasmid cDNA clones, the inserts PCR amplified, then denatured (using dimethyl sulfoxide) and spotted onto the surface using a robotic device. About 20,000 spots can be printed per glass slide, with each spot able to be of a whole gene length
- Photolithographic arrays: use a quartz wafer used as platform for printing oligonucleotides (20-25 mer), and allow making of ultra-high density arrays with millions of spots printed per slide

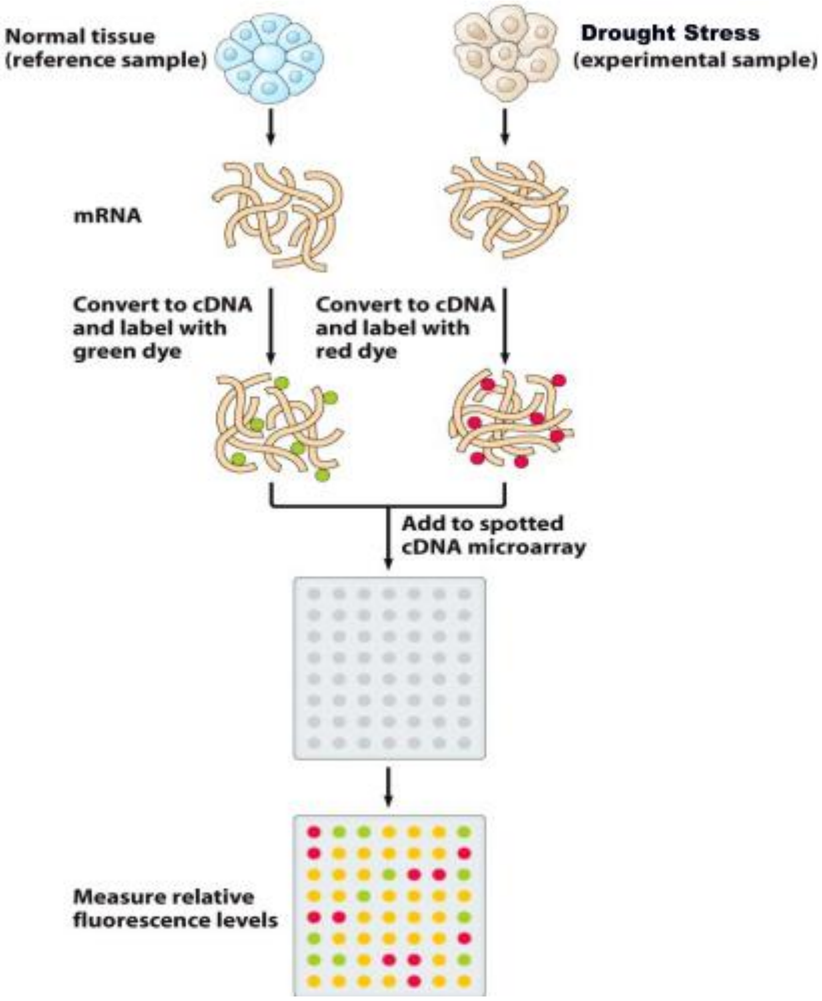

Preparation of target DNA
Gene amplification
Print DNA
False colour image of scanned slide
Hybridize
Preparation of fluorescent probe
mRNA 1
AAAAAAAAAAAA
TTTTTTTTT
mRNA 2
AAAAAAAAAAAA
TTTTTTTTT
Fluorescent cDNA synthesis





Using microarrays to study stress resistance

- Usually studies are designed to be able to compare a treatment to a control condition, tagged with different fluorescent dyes (e.g. Cy3 for green and Cy5 for red)


Normal tissue
(reference sample)
Drought Stress
(experimental sample)
mRNA
Convert to cDNA and label with green dye
Convert to cDNA and label with red dye
Add to spotted cDNA microarray
Measure relative fluorescence levels

Drought tolerance mechanisms

- Delay of senescence: in order to avoid plant death longer to cope with the stressor, involves repression of senescence-associated protein DIN1, auxin-repressive protein, magnesium chelatase, and ubiquitins
- Transport facilitation: to offset reduced water access, involve induction of lipid-transfer protein and protein-transport proteins, with suppression of aquaporins, DNA-J like protein-susceptibility
- Promotion of pollen tube growth: induction of RAC-GTP binding protein
- Reduced energy capture and CO2 fixation: to avoid growth while stressed, involves closure of stomata, repression of ATP synthase, repression of NADH ubiquinone oxidoreductase and NADH dehydrogenase
- Pathogenesis-related genes: repression of the pathogenesis-related proteins is associated with drought tolerance, while repression of disease-resistance response proteins is associated with drought susceptibility

Cold tolerance mechanisms

- Stress perception: high-repression (>5-fold) of membrane-related protein CP5 in the susceptible genotypes so that plants ‘feel the cold less’

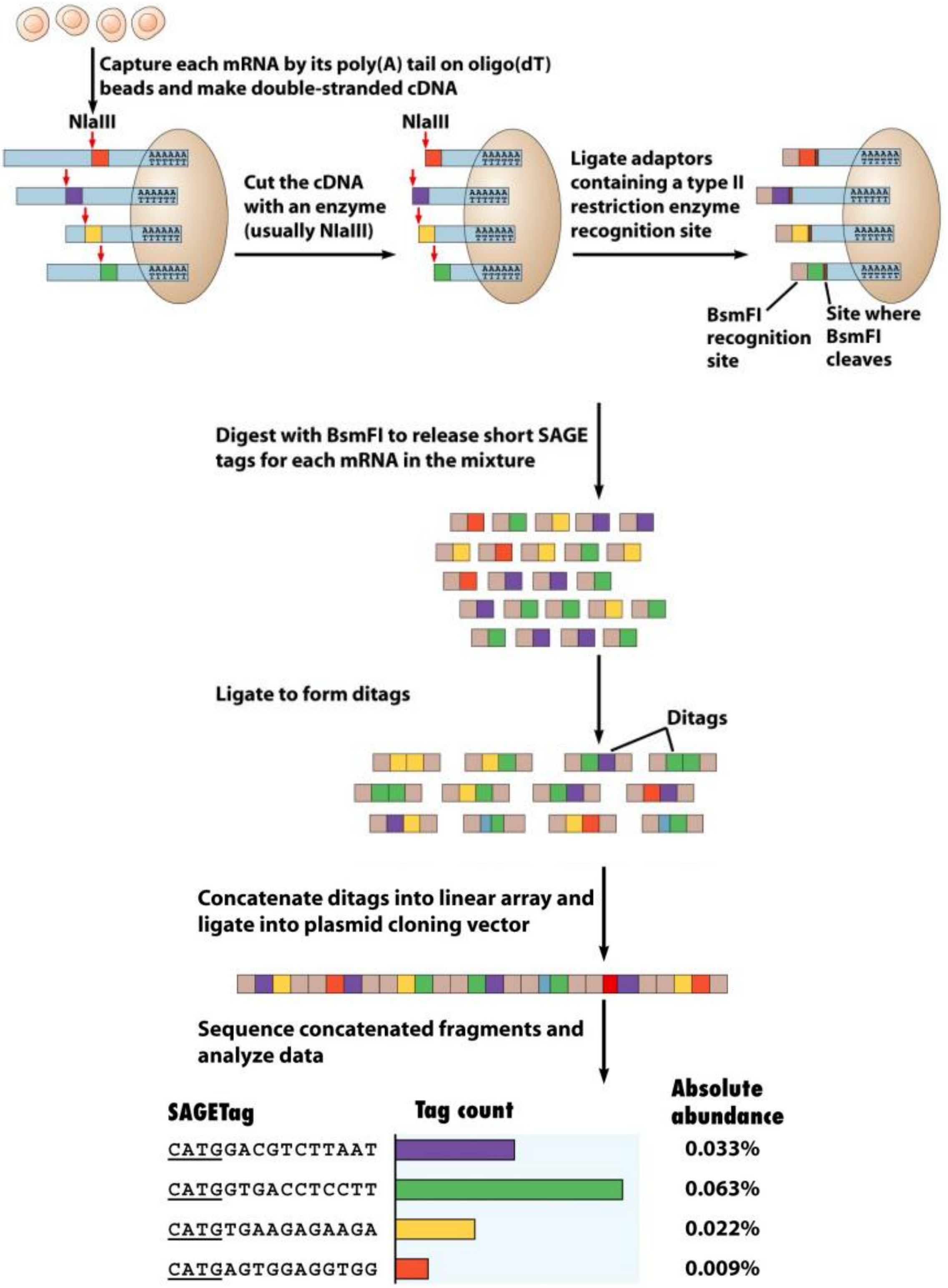
- Ca 2+ signalling: repression of the Ca-binding mitochondrial carrier to reduce calcium efflux from the cell, and thereby increasing cell signalling
- Cold adaptation (regulation of ICE1): repression of ubiquitins and polyubiquitins leading to induction of ICE1, associated with cold tolerance
- Accumulation of osmolytes: accumulation of polyamines by induction of S-adenosylmethionine decarboxylase; the accumulation of disaccharides, especially sucrose, in the leaves by suppression of β-glucosidase and β-galactosidase, and induction of sucrose synthase, to help maintain cell turgidity thereby protecting the cell
- Flower abortion (associated with susceptibility): accumulation of sucrose in chickpea flowers by induction of α-amylase precursor and α-amylase genes, which makes flowers sterile
- Delay of senescence: repression of senescence-associated protein DIN1 and auxin-repressed protein
- Suppression of transportation (associated with susceptibility): repression of sorting nexin protein that controls trafficking of membrane/secretory proteins

Salt tolerance mechanisms

- Ca 2+ influx: repression of the Ca-binding mitochondrial carrier in the roots of tolerant genotypes to help maintain ion and pH balance
- Ion homeostasis and/or pH balance: repression of carbonic anhydrase
- Suppression of aquaporins: to regulate the salt uptake; early repression may be associated with salt tolerance
- Suppression of lignification: freeing up cellular resources that can be used in other processes by repressing repression of glycine-rich proteins
- Delay of senescence: repression of senescence-associated proteins, ripening-related protein, and ubiquitin-conjugating protein associated with photomorphogenesis
- Accumulation of osmolytes: accumulate osmolytes like sucrose and proline by repressing β-galactosidase and proline oxidase
- Energy utilisation: epression of fructose 1,6-bisphosphatase (and thus gluconeogenesis)
- Pathogenesis-related mechanisms: high-induction of pathogenesis-related protein 4A in response to high-salinity stress

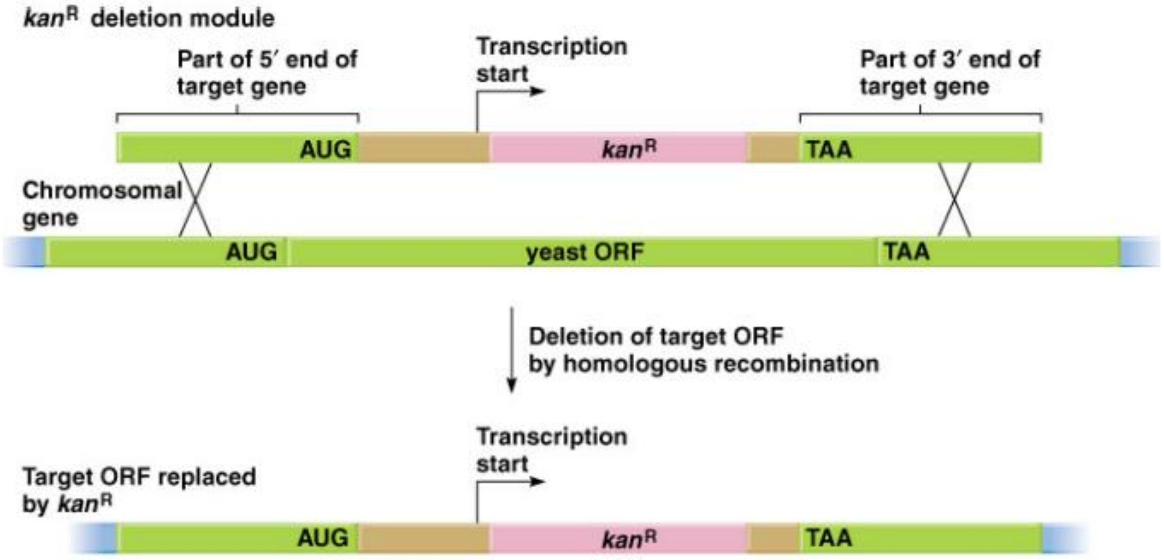
Serial Analysis of Gene Expression

- SAGE is an open architecture system where plasmid libraries are made from the mRNA poll of a cell and large numbers of clones are sequenced
- The number of times a sequence is present for a particular gene is an indication of its abundance in original mRNA pool
- First, polyadenylated mRNAs are captured by binding them to oligo(dT) containing beads
- mRNAs are converted to cDNAs with RT, then the cDNA are cleaved with cutters like NlaIII
- Short linkers are added containing another restriction site (BsmFI), but this one cuts several bases away from the recognition site
- Cutting with this second enzyme produces short DNA tags which are ligated to form dotages, and then concatenated into a linear array and sequenced
- The abundance of each tag is determined, thereby giving an indication of how strongly expressed each associated gene is


Capture each mRNA by its poly(A) tail on oligo(dT) beads and make double-stranded cDNA
NlaIII
NlaIII
Cut the cDNA with an enzyme (usually NlaIII)
Ligate adaptors containing a type II restriction enzyme recognition site
BsmFI recognition site
Site where BsmFI cleaves
Digest with BsmFI to release short SAGE tags for each mRNA in the mixture
Ligate to form ditags
Ditags
Concatenate ditags into linear array and ligate into plasmid cloning vector
Sequence concatenated fragments and analyze data
SAGETag
Tag count
Absolute abundance
CATGGACGTCTTAAT
CATGGTGACCTCCTT
CATGTGAAGAGAAGA
CATGAGTGGAGGTGG
0.033%
0.063%
0.022%
0.009%

Confirmation of gene function

- After identification of a correlation in microarray studies, it is necessary to make a confirmation of gene function by real-time PCR, gene knockouts, TILLING mutants, or over-expressing transgenics
- To create a knockout in yeast, the complete sequence of the gene is needed so that a region to be inserted can be created with the correct complementary regions
- Yeast cells carrying the artificial module (deleted ORF) can be selected by screening for inserted marker (e.g. growing on kanamycin)


kanR deletion module
Part of 5′ end of target gene
Transcription start
Part of 3′ end of target gene
AUG
kanR
TAA
Chromosomal gene
AUG
yeast ORF
TAA
Deletion of target ORF by homologous recombination
Transcription start
Target ORF replaced by kanR
AUG
kanR
TAA

Creating transgenic plants

- ‘Gene-of-interest’ (GOI) is ligated into bacterial T-DNA (tumor-inducing DNA) under the control of a specific promoter
- The promoter used can be constitutive (expressed normally) or stress-induced (activates under stress)
- T-DNA vector containing the gene of interest is transformed into plant cells via Agrobacterium tumefaciens
- Transformed plant cells are grown to full plants (transgenics) using micro-propagation techniques, and then screened for gene expression and stress tolerance

Transgenic chickpea example

- They transformed chickpea with two different genes
- First transgenic was transformed with a functional gene P5CSF129A, under control of a constitutive promoter. This gene codes for proline, an osmolyte that protects plants under drought stress
- Second transgenic was transformed with a regulatory gene DREB1A,under control of a stress-inducible promoter from the rd29A gene. This gene is a transcription factor known to activate downstream drought tolerance genes
- Physiological parameters were studied in transgenic generations (T2 to T4)
- The transgenics with both the constructs showed a decline in the transpiration rate in drier soils, thus increasing water use efficiency under stress
- Overexpression of P5CSF129A gene produced more proline that helped these plants maintain cell turgor and physiological processes under drought stress. This resulted in postponement of dehydration as water deficit developed under drought stress.
- Hence it was concluded that although both constructs conferred drought tolerance, the use of transcription factors driven by stress-responsive promoters is a more promising approach to develop drought tolerant transgenics

Sequencing Methods

Overview

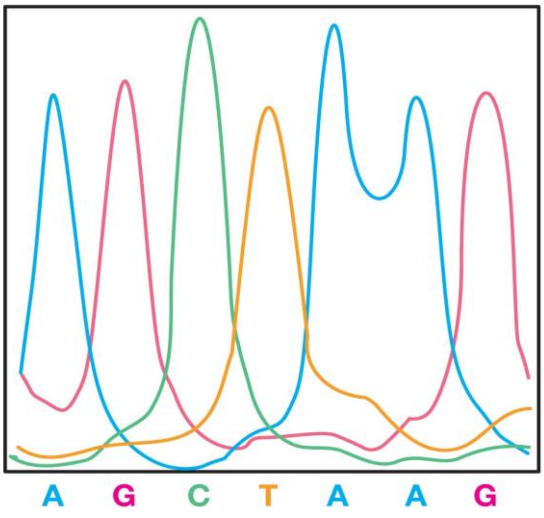
- DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule
- A genome includes the entire complement of genetic information, including genes, regulatory sequences, and noncoding DNA
- Sanger sequenced the first genome, which was the Phi X 174 (a bacteriophage)

Sanger sequencing

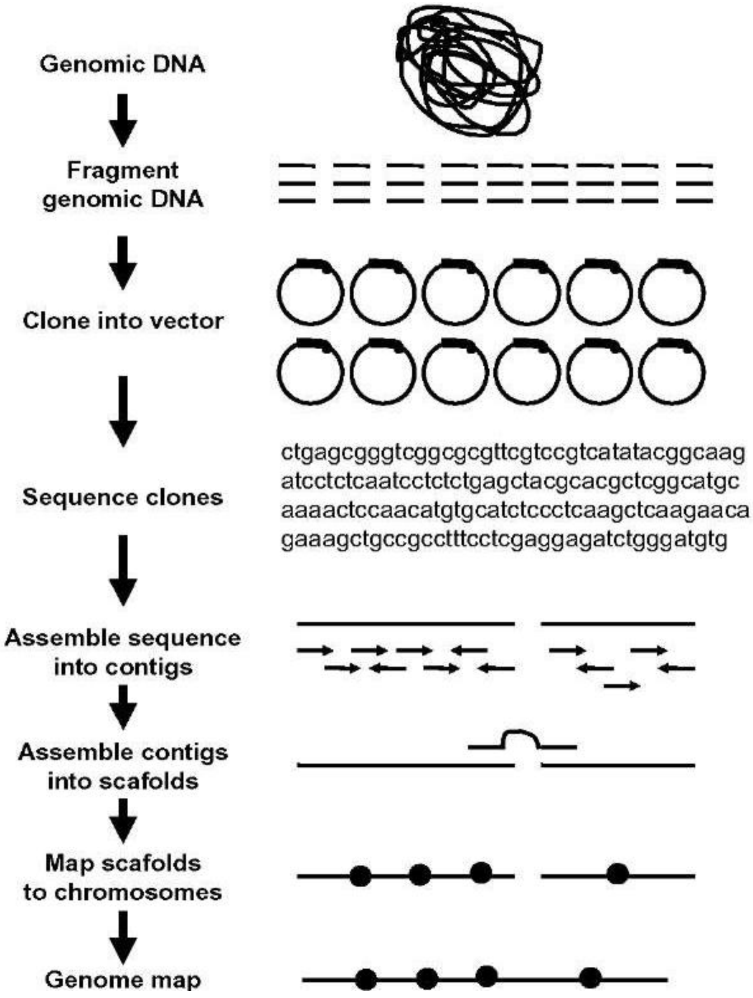
- Invented by Nobel Prize winner Fred Sanger
- Dideoxy analogs of dNTPs used in conjunction with dNTPs (Figure1)
- Analog prevents further extension of DNA chain (Figure 2)
- Bases are labelled with radioactivity
- Gel electrophoresis is then performed on products (Figure 3)

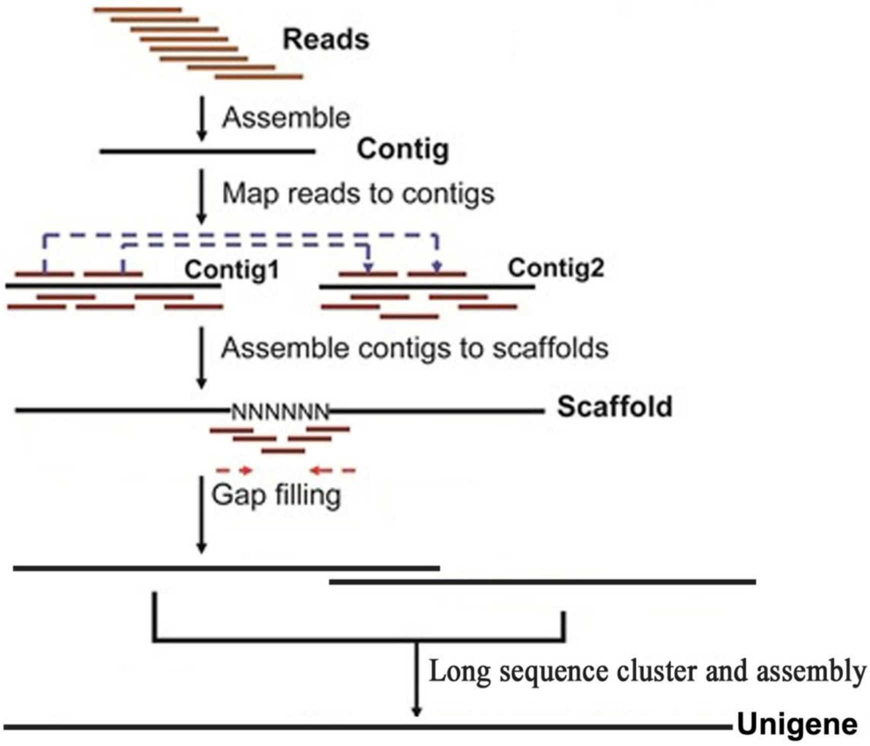
Whole genome sequencing

- Large-scale sequencing projects have led to automated DNA sequencing systems, based on automated Sanger sequencing and replacing radioactivity by florescent dyes
- Shotgun sequencing is a technique that involves plasmid extraction and bacterial colony picking which makes this method quite cumbersome
- Second generation DNA sequencing is much faster (100 times) than Sanger sequencing
- It uses massively parallel methods which use large number of samples sequenced side by side and increased computer power and miniaturization
    - 454 Life Sciences pyrosequencing
    - Illumina/Solexa sequencing
    - ABi SOLiD sequencing
- One of the biggest ongoing challenges for these methods is in read length, and data storage and analysis



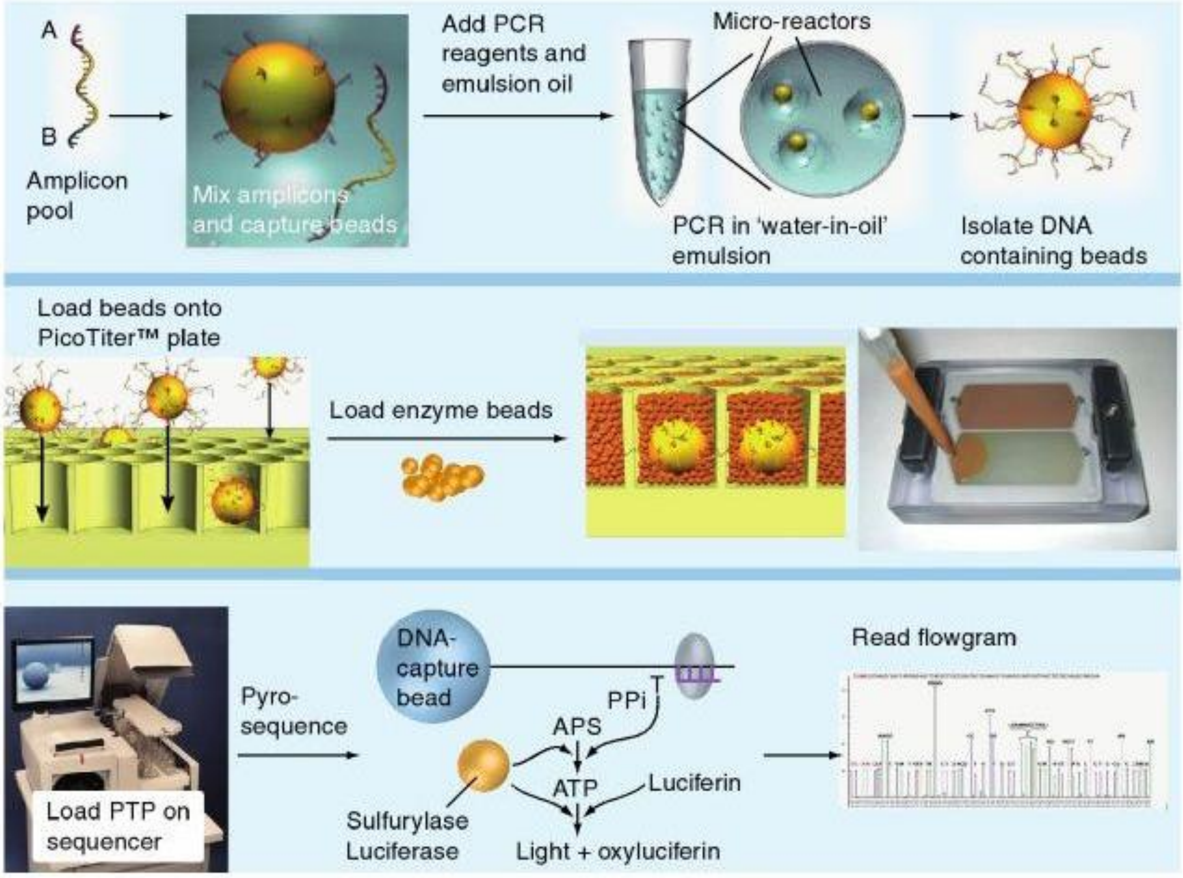
In automated sequencing, each base has its own fluoresent dye.

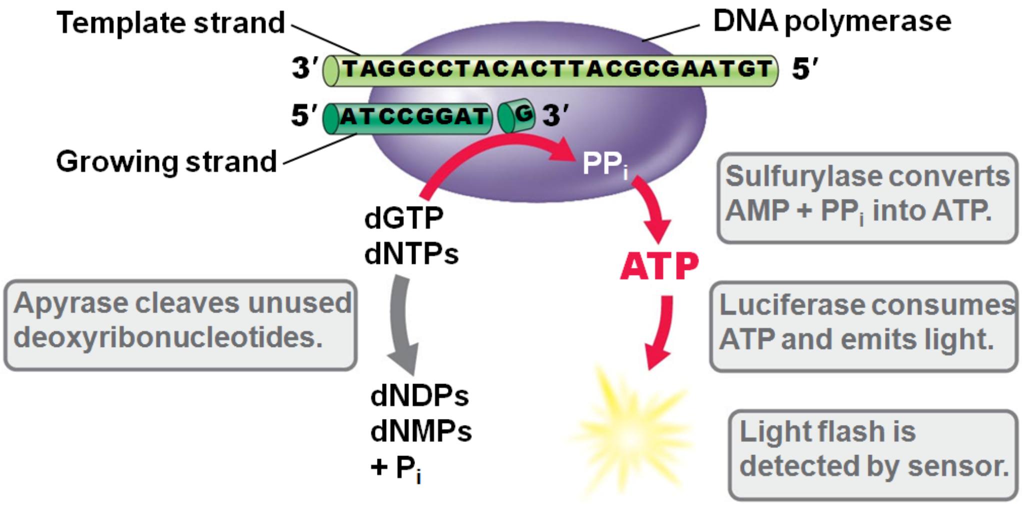

Genomic DNA
Fragment genomic DNA
Clone into vector
Sequence clones
ctgagcgggtcggcgcgttcgtccgtcatatacggcaag
atcctctcaatcctctctgagctacgcacgctcggcatgc
aaaactccaacatgtgcatctccctcaagctcaagaaca
gaaagctgccgcctttcctcgaggagatctgggatgtg
Assemble sequence into contigs
Assemble contigs into scafolds
Map scafolds to chromosomes
Genome map


Reads
Assemble
Contig
Map reads to contigs
Contig1
Contig2
Assemble contigs to scaffolds
NNNNNN
Scaffold
Gap filling
Long sequence cluster and assembly
Unigene

Roche 454 Pyrosequencing

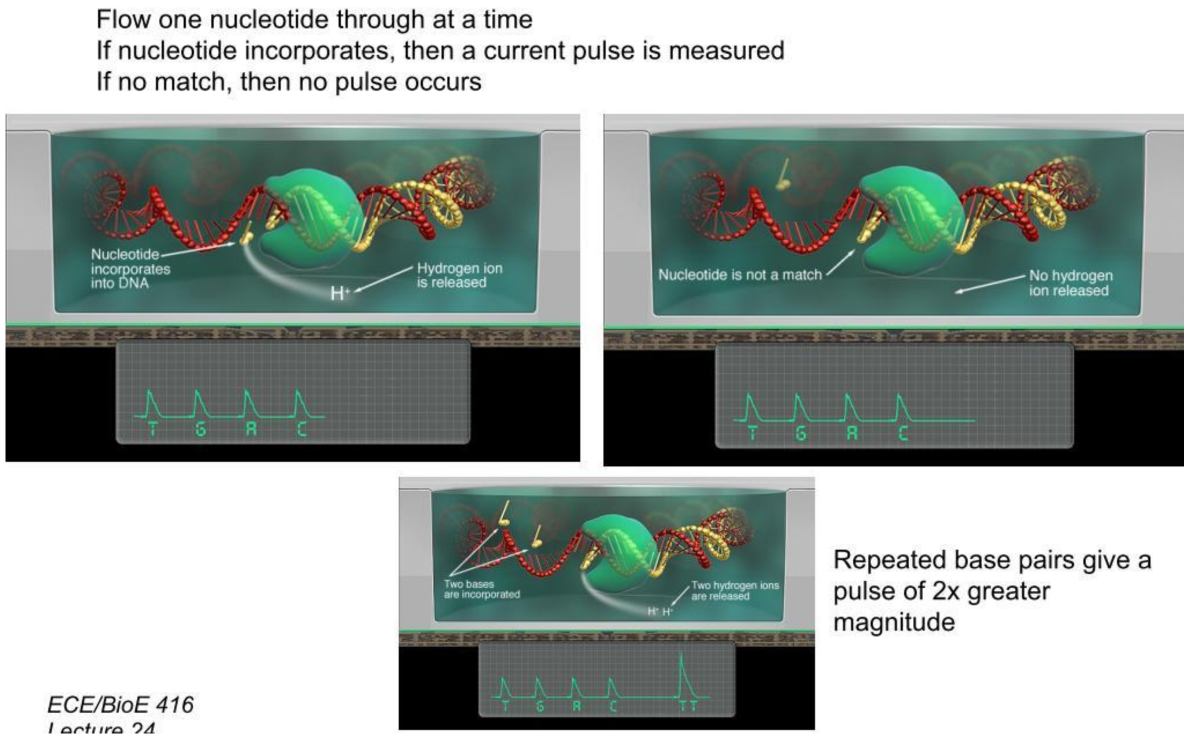
- Pyrosequencing is a method of DNA sequencing based on a sequencing-by-synthesis principle
- It differs from Sanger sequencing in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides
- First, DNA is broken into small segments, and amplified using a polymerase chain reaction
- During sequencing, light is released each time a base is added to DNA strand
- The instrument actually measures release of light, with one type of base added at a time so we can determine which base was included
- Can handle only short stretches of DNA




Template strand
DNA polymerase
3′
TAGGCCTACACTTACGCGAATGT
5′
5′
ATCCGGAT
G
3′
Growing strand
PP_i
dGTP
dNTPs
ATP
Sulfurylase converts AMP + PP_i into ATP.
Apyrase cleaves unused deoxyribonucleotides.
Luciferase consumes ATP and emits light.
dNDPs
dNMPs
+ P_i
Light flash is detected by sensor.

Ion torrent

- When a nucleotide is incorporated into the growing strand, a H+ ion is released, which changes the surrounding pH and thus is detected by the semiconductor
- Sequencing is therefore a sequential flow of single bases over the semicondutor chip whilst measuring the pH as each nucleotide is introduced one at a time
- If there are two identical bases on the DNA strand, the voltage will be double, and the chip will record two identical bases



Flow one nucleotide through at a time
If nucleotide incorporates, then a current pulse is measured
If no match, then no pulse occurs

Repeated base pairs give a pulse of 2x greater magnitude

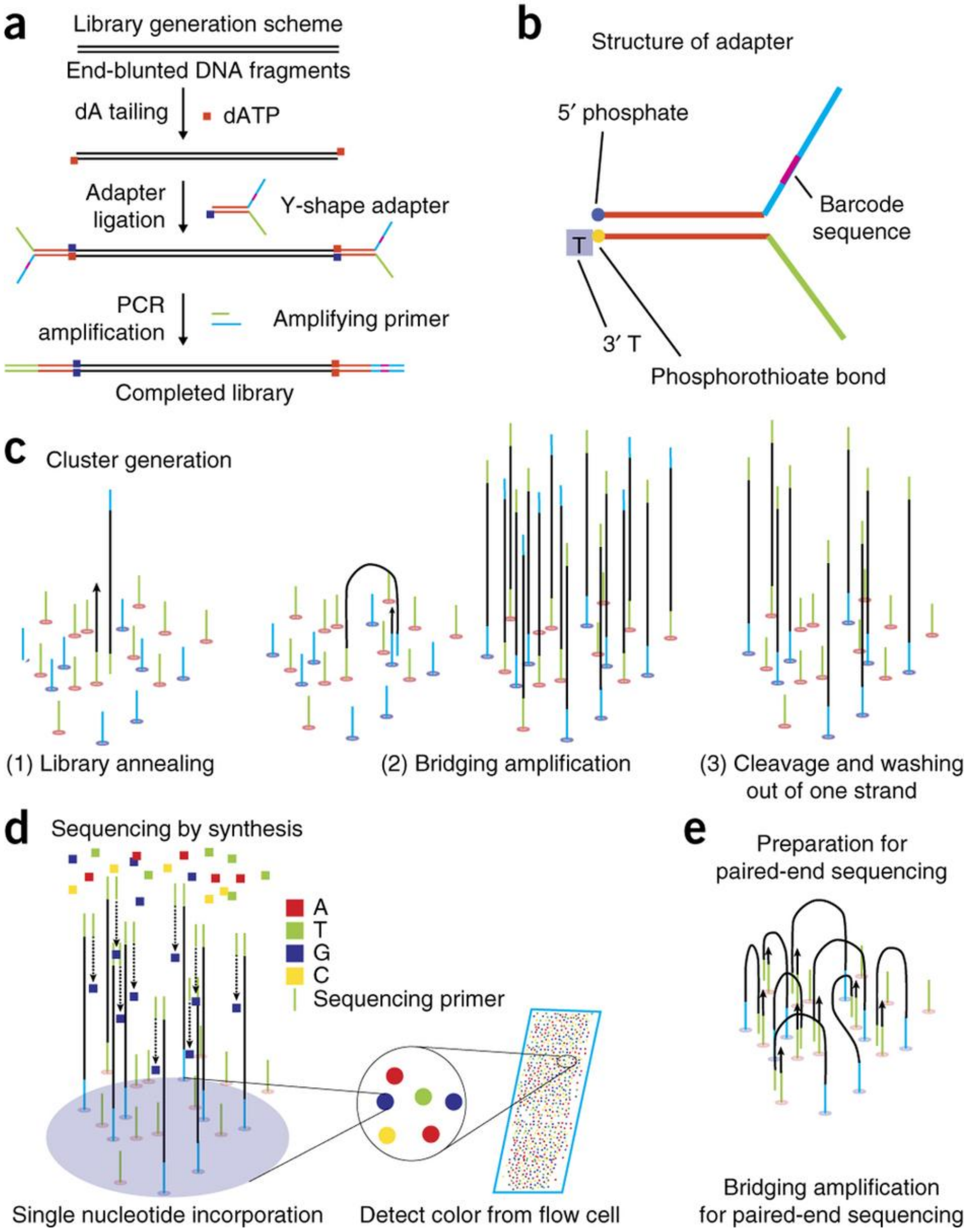
ECE/BioE 416
Lecture 24

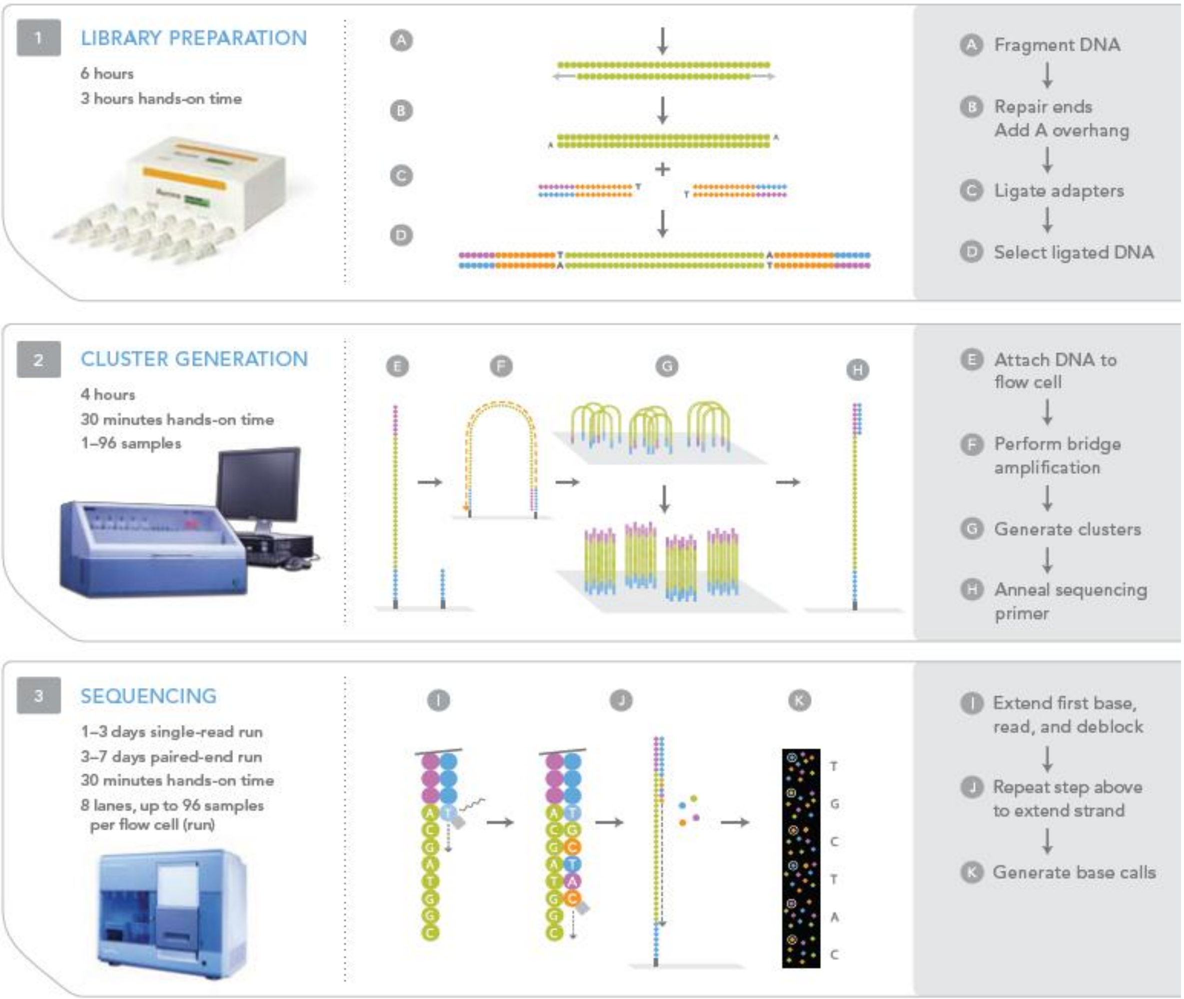
Nucleotide incorporates into DNA
Hydrogen ion is released
H+
T G A C
Nucleotide is not a match
No hydrogen ion released
T G A C
Two bases are incorporated
Two hydrogen ions are released
H+ H+
T G A C TT

Illumina

- Illumina sequencing technology works in three basic steps: amplify, sequence, and analyse
- The process begins with purified DNA. The DNA gets chopped up into smaller pieces and given adapters, indices, and other kinds of molecular modifications that act as reference points during amplification, sequencing, and analysis
- The modified DNA is loaded onto a specialized chip where amplification and sequencing will take place
- Along the bottom of the chip are hundreds of thousands of oligonucleotides which are anchored to the chip and able to grab DNA fragments that have complementary sequences
- Once the fragments have attached, a phase called cluster generation begins. This step makes about a thousand copies of each fragment of DNA
- Next, primers and modified nucleotides enter the chip. These nucleotides have reversible 3' blockers that force the primers to add on only one nucleotide at a time as well as fluorescent tags. After each round of synthesis, a camera takes a picture of the chip
- A computer determines what base was added by the wavelength of the fluorescent tag and records it for every spot on the chip. After each round, non-incorporated molecules are washed away
- A chemical deblocking step is then used in the removal of the 3’ terminal blocking group and the dye in a single step. The process continues until the full DNA molecule is sequenced

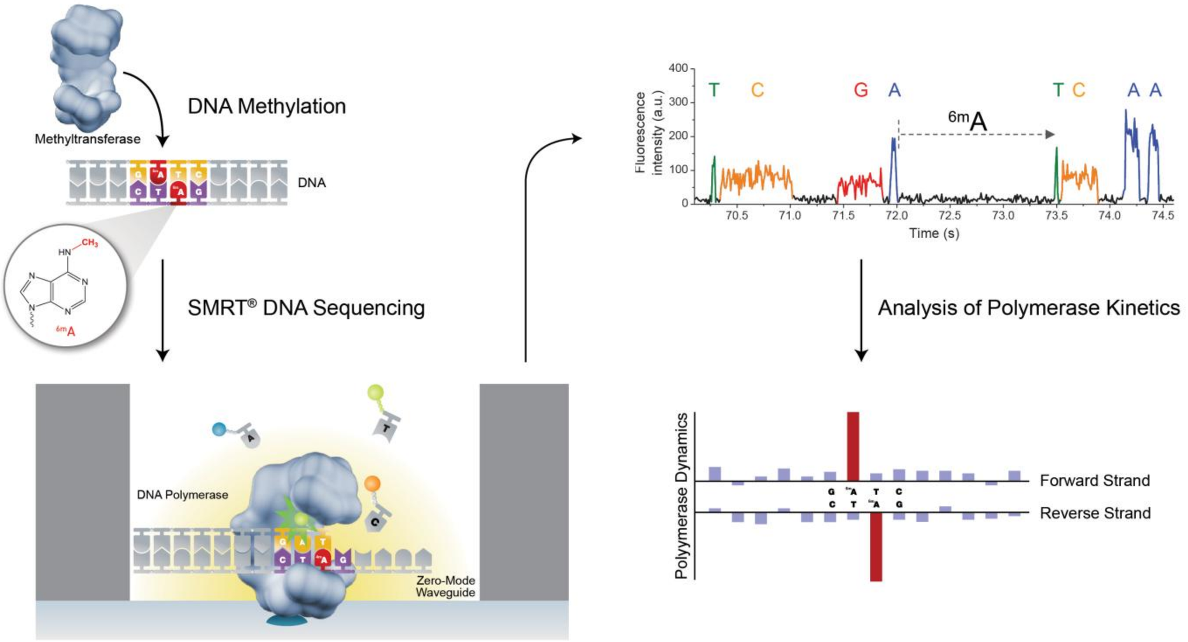
- With this technology, thousands of places throughout the genome are sequenced at once via massive parallel sequencing
- Illumina sells a wide range of sequencing, including the MiSeq series for smaller scale work, HiSeq for medium scale work, and the HiSeq X for full genome sequencing


a
Library generation scheme
End-blunted DNA fragments
dA tailing
dATP
Adapter ligation
Y-shape adapter
PCR amplification
Amplifying primer
Completed library
b
Structure of adapter
5′ phosphate
Barcode sequence
T
3′ T
Phosphorothioate bond
c
Cluster generation
(1) Library annealing
(2) Bridging amplification
(3) Cleavage and washing out of one strand
d
Sequencing by synthesis
A
T
G
C
Sequencing primer
Single nucleotide incorporation
Detect color from flow cell
e
Preparation for paired-end sequencing
Bridging amplification for paired-end sequencing



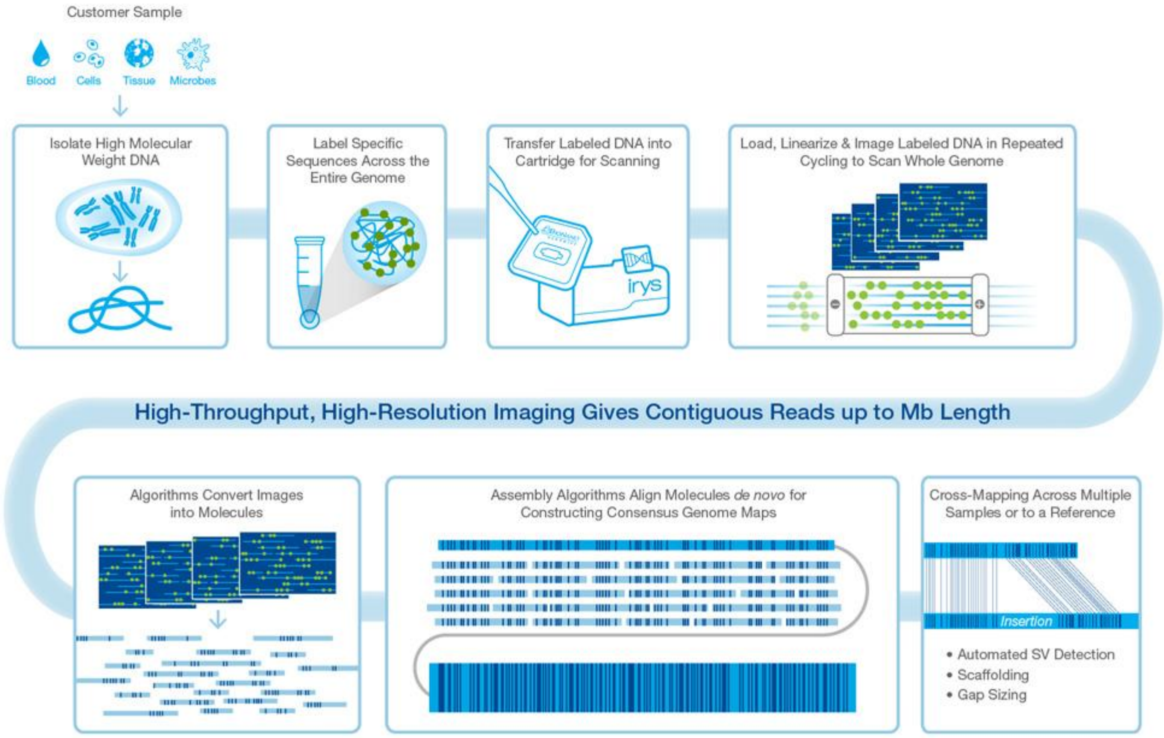
Pacific biosciences

- Single Molecule Real Time sequencing involves reactions carried out in nanocontainers - 20zl sequencing reactions, with single-stranded DNA fragments attached
- Single molecule real time sequencing (SMRT) is a parallelized single molecule DNA sequencing method
- Single molecule real time sequencing utilizes a zero-mode waveguide (ZMW), which is a structure that creates an illuminated observation volume that is small enough to observe only a single nucleotide of DNA being incorporated by DNA polymerase
- A single DNA polymerase enzyme is affixed at the bottom of a ZMW with a single molecule of DNA as a template
- Each of the four DNA bases is attached to one of four different fluorescent dyes. When a nucleotide is incorporated by the DNA polymerase, the fluorescent tag is cleaved off and diffuses out of the observation area of the ZMW where its fluorescence is no longer observable
- A detector detects the fluorescent signal of the nucleotide incorporation, and the base call is made according to the corresponding fluorescence of the dye



BioNano optical mapping

- Optical mapping is a technique for constructing ordered, genome-wide, high-resolution restriction maps from single, stained molecules of DNA, called "optical maps"
- By mapping the location of restriction enzyme sites along the unknown DNA of an organism, the spectrum of resulting DNA fragments collectively serves as a unique "fingerprint" or "barcode" for that sequence
- This method has since been integral to the assembly process of many large-scale sequencing projects for both microbial and eukaryotic genomes
- Genomic DNA is obtained from lysed cells, and randomly sheared to produce a "library" of large genomic molecules for optical mapping
- A single molecule of DNA is stretched (or elongated) and held in place on a slide under a fluorescent microscope due to charge interactions
- The DNA molecule is digested by restriction enzymes, and the resulting molecule fragments remain attached to the surface
- The fragment ends at the cleavage sites are drawn back (due to elasticity of linearized DNA), leaving gaps which are identifiable under the microscope
- DNA fragments stained with intercalating dye are visualized by fluorescence microscopy and are sized by measuring the integrated fluorescence intensity. This produces an optical map of single molecules
- Individual optical maps are combined to produce a consensus, genomic optical map

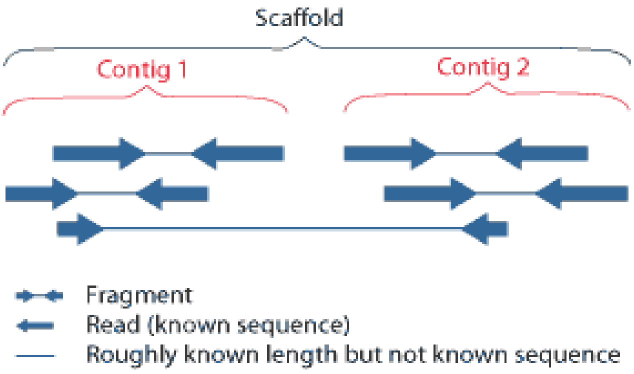

Customer Sample
Blood
Cells
Tissue
Microbes
Isolate High Molecular Weight DNA
Label Specific Sequences Across the Entire Genome
Transfer Labeled DNA into Cartridge for Scanning
irys
Load, Linearize & Image Labeled DNA in Repeated Cycling to Scan Whole Genome
High-Throughput, High-Resolution Imaging Gives Contiguous Reads up to Mb Length
Algorithms Convert Images into Molecules
Assembly Algorithms Align Molecules de novo for Constructing Consensus Genome Maps
Cross-Mapping Across Multiple Samples or to a Reference
Insertion
• Automated SV Detection
• Scaffolding
• Gap Sizing

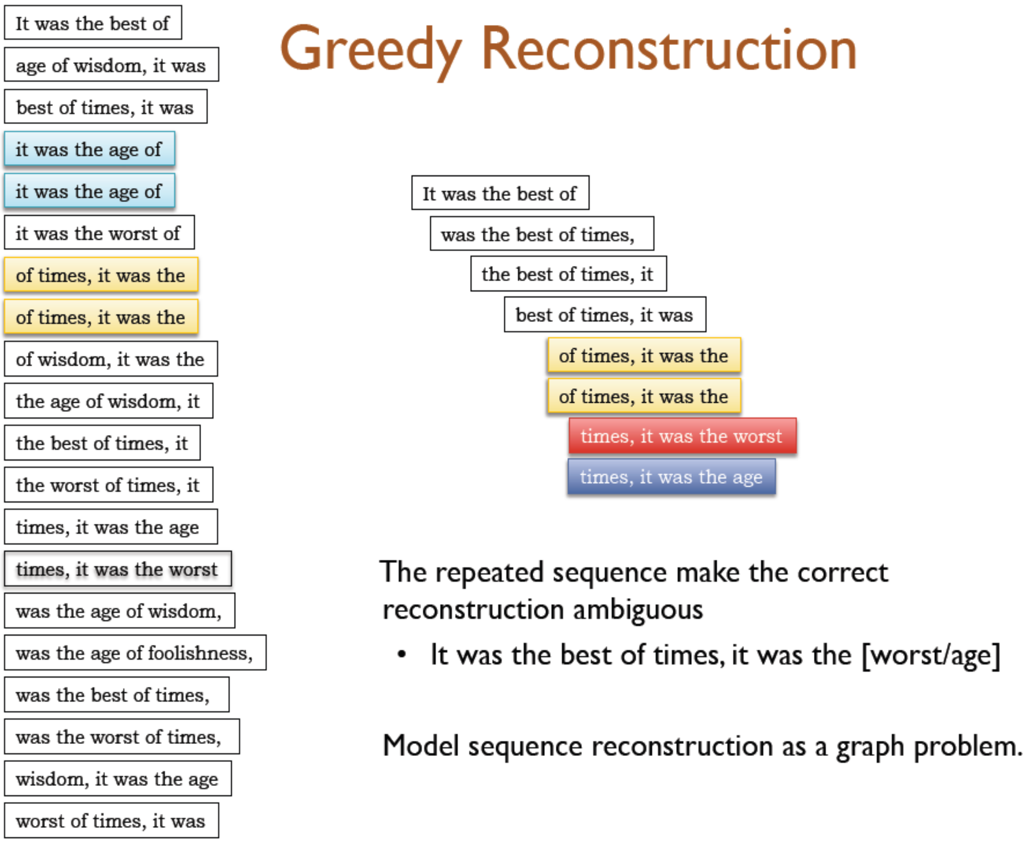
Multiplexing

- Multiplexing refers to any method of attaching a bar code before the sequencing
- Large adapted primers are used to barcode the sample
- The first five bases in an illumina run will be used for identification of the clusters, assumes random base order – must be considered when designing adaptors

DNA sequence assembly

- Genome assembly consists of connecting the DNA fragments in the correct order
- De Brujin graphs are used for assembling genomic sequences. An *n*-dimensional De Bruijn graph of *m* symbols is a directed graph representing overlaps between sequences of symbols
- The Burrows–Wheeler transform rearranges a character string into runs of similar characters. This is useful for compression, since it tends to be easy to compress a string that has runs of repeated characters. The transformation is reversible, so it is a "free" method of improving the efficiency of text compression algorithms, costing only some computation
- Short sequence mapping tools such as Bow Tie, BWA, and Novoalign are designed for short read assembly. They use Burrows-Wheeler indexing purpose-built for a small computational footprint for DNA sequence alignment





Greedy Reconstruction

It was the best of
age of wisdom, it was
best of times, it was
it was the age of
it was the age of
it was the worst of
of times, it was the
of times, it was the
of wisdom, it was the
the age of wisdom, it
the best of times, it
the worst of times, it
times, it was the age
times, it was the worst
was the age of wisdom,
was the age of foolishness,
was the best of times,
was the worst of times,
wisdom, it was the age
worst of times, it was

It was the best of
was the best of times,
the best of times, it
best of times, it was
of times, it was the
of times, it was the
times, it was the worst
times, it was the age

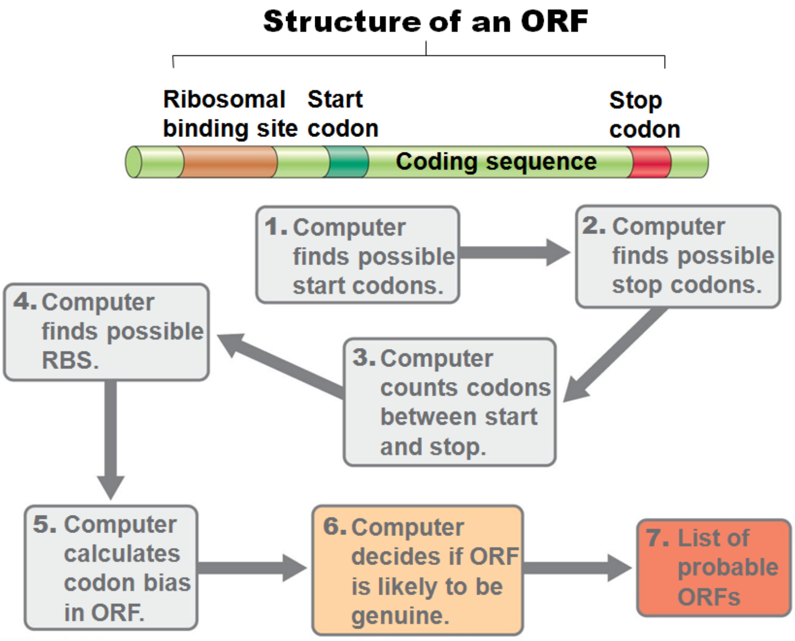
The repeated sequence make the correct reconstruction ambiguous

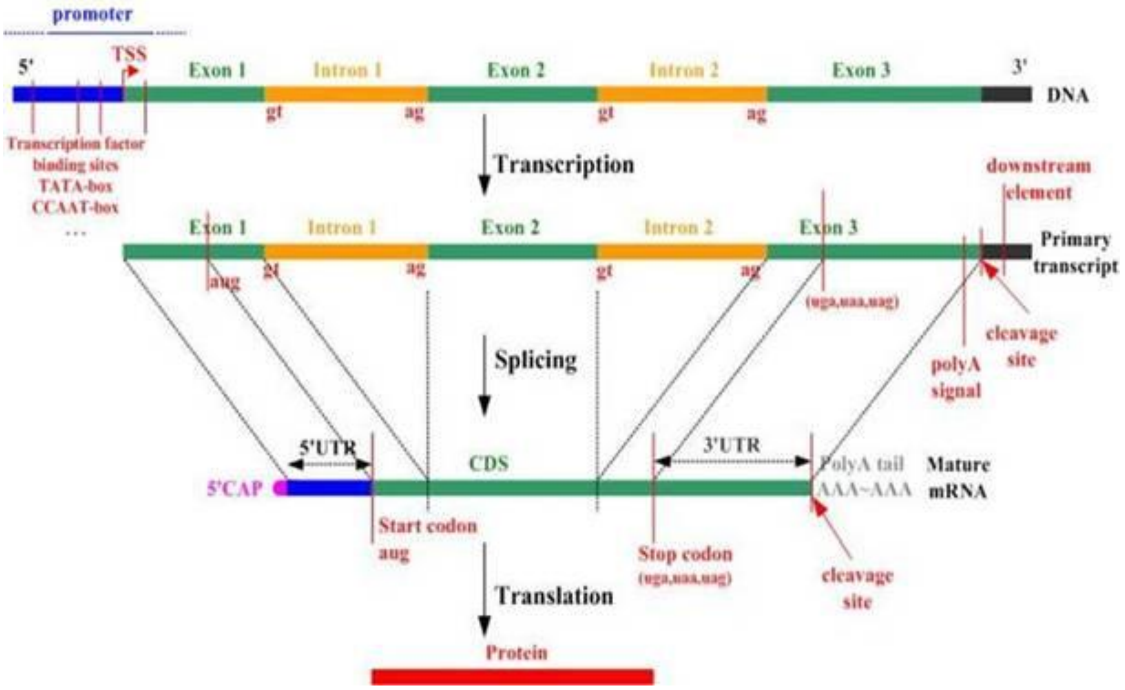
- It was the best of times, it was the [worst/age]

Model sequence reconstruction as a graph problem.

Annotating the genome

- Annotating the genome refers to the process of converting raw sequence data into a list of genes present in the genome
- Annotation is the major bottleneck in genomics
- Computer algorithms are used to search for open reading frames by looking for start/stop codons and Shine–Dalgarno sequences (ribosomal binding sites)
- Hypothetical proteins are uncharacterized ORFs; proteins that likely exist but whose function is currently unknown


Structure of an ORF
Ribosomal binding site
Start codon
Stop codon
Coding sequence
1. Computer finds possible start codons.
2. Computer finds possible stop codons.
3. Computer counts codons between start and stop.
4. Computer finds possible RBS.
5. Computer calculates codon bias in ORF.
6. Computer decides if ORF is likely to be genuine.
7. List of probable ORFs



Genome size and content

- *Archaea* typically devote a higher percentage of their genomes to energy and coenzyme production than do *Bacteria*
- *Archaea* also contain fewer genes for carbohydrate metabolism or cytoplasmic membrane functions than do *Bacteria*
- Some mitochondria and some protozoa use variation of genetic code

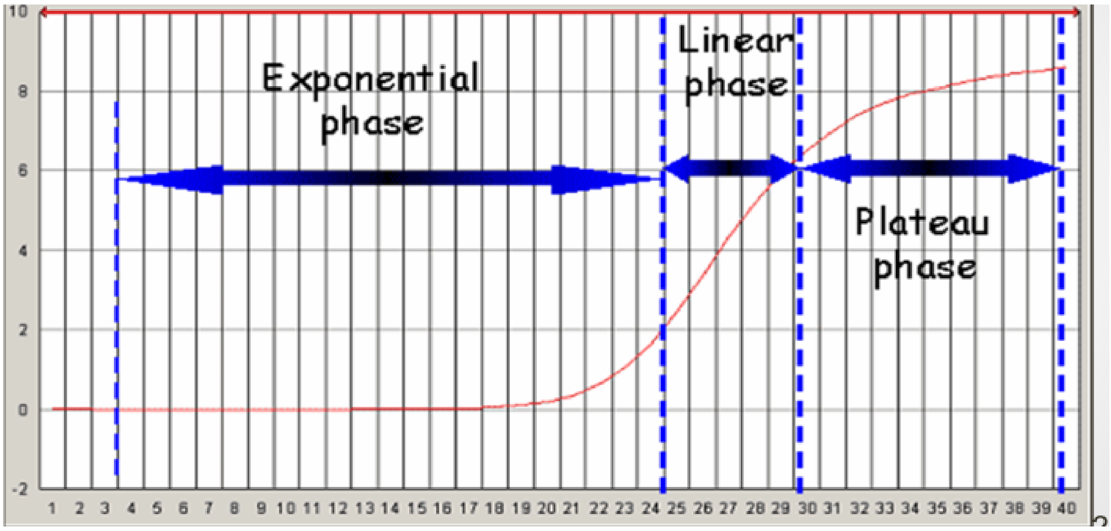
Quantitative PCR

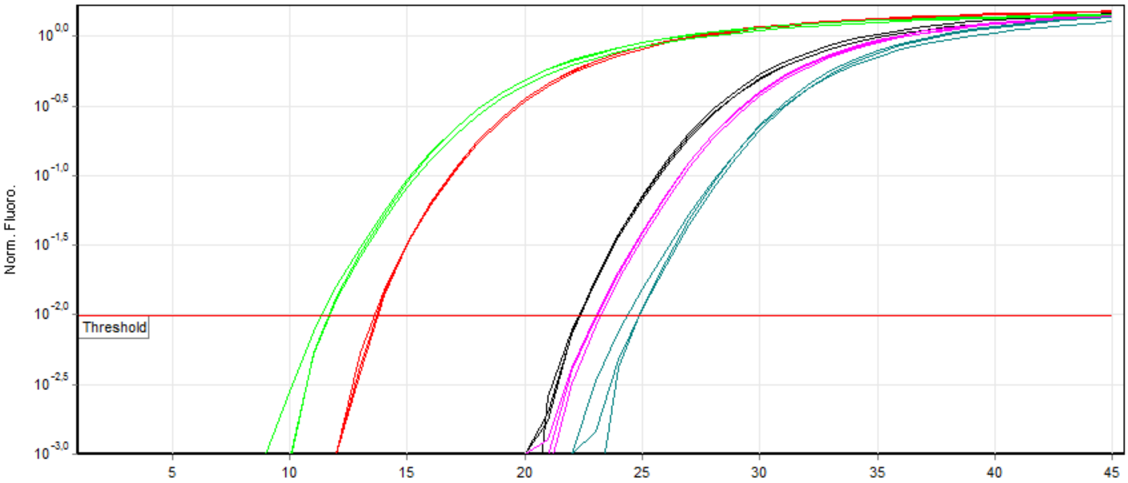
Reaction methods

- A real-time polymerase chain reaction is a technique based on the polymerase chain reaction
- It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR
- Two common methods for the detection of PCR products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe
- The PCR reaction amplifies according to the equation:

$$X_{n+1} = X_0(1 + \mathrm{Eff})n$$

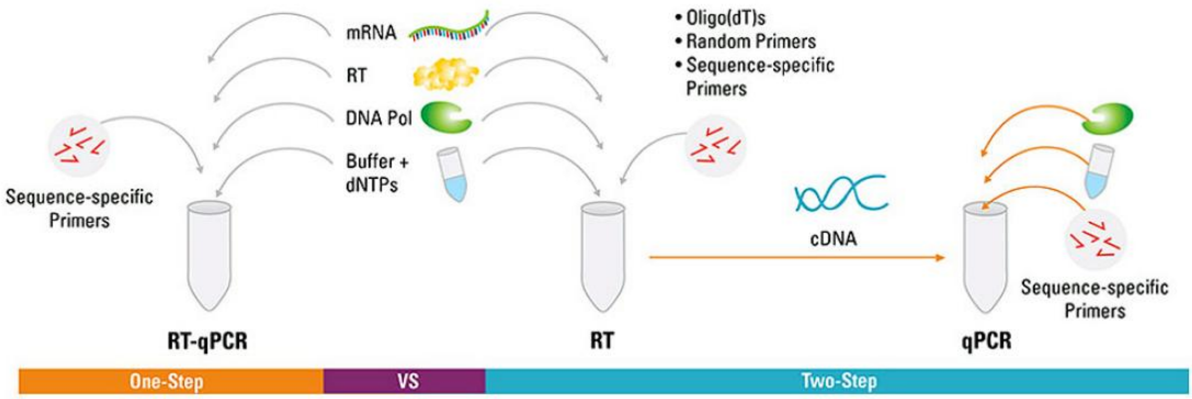
- qPCR works by identifying the number of cycles (Ct value) required to reach a pre-defined threshold of Relative Fluorescent Units
- The lower is the Ct value, the more DNA was originally present in the sample

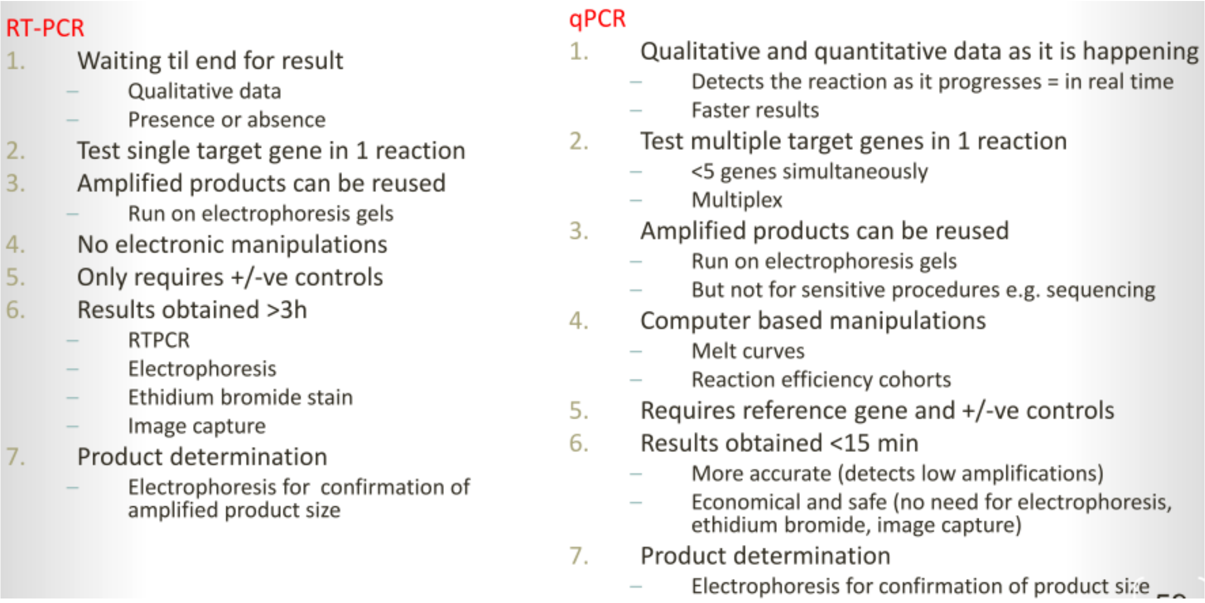




One step vs two step methods

- There are different ways of conducting a qPCR analysis – one using a single step and a single reaction, and the other involving a two-step process
- The one-step process has fewer chances for contamination or error, however the two-step process takes advantage of the fact that cDNA is much more stable than RNA
- This also means that the two-step process can begin with smaller initial concentrations of RNA



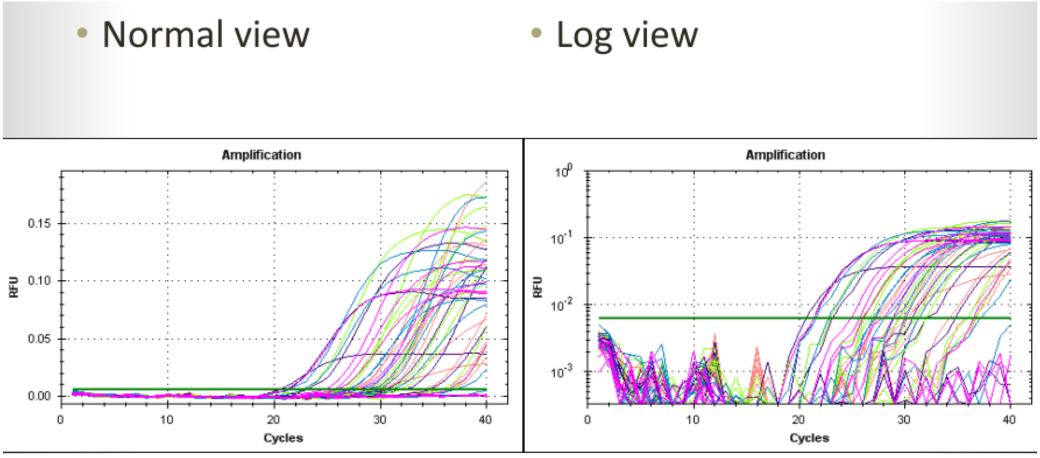


RT-PCR

1. Waiting til end for result
   - Qualitative data
   - Presence or absence
2. Test single target gene in 1 reaction
3. Amplified products can be reused
   - Run on electrophoresis gels
4. No electronic manipulations
5. Only requires +/-ve controls
6. Results obtained >3h
   - RTPCR
   - Electrophoresis
   - Ethidium bromide stain
   - Image capture
7. Product determination
   - Electrophoresis for confirmation of amplified product size

qPCR

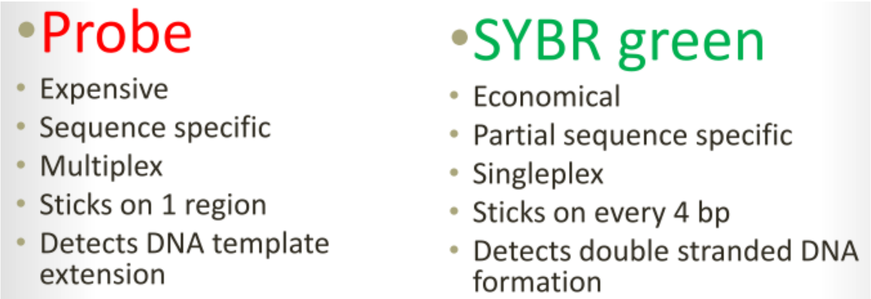
1. Qualitative and quantitative data as it is happening
   - Detects the reaction as it progresses = in real time
   - Faster results
2. Test multiple target genes in 1 reaction
   - <5 genes simultaneously
   - Multiplex
3. Amplified products can be reused
   - Run on electrophoresis gels
   - But not for sensitive procedures e.g. sequencing
4. Computer based manipulations
   - Melt curves
   - Reaction efficiency cohorts
5. Requires reference gene and +/-ve controls
6. Results obtained <15 min
   - More accurate (detects low amplifications)
   - Economical and safe (no need for electrophoresis, ethidium bromide, image capture)
7. Product determination
   - Electrophoresis for confirmation of product size



- Normal view
- Log view

Primers and detection agents

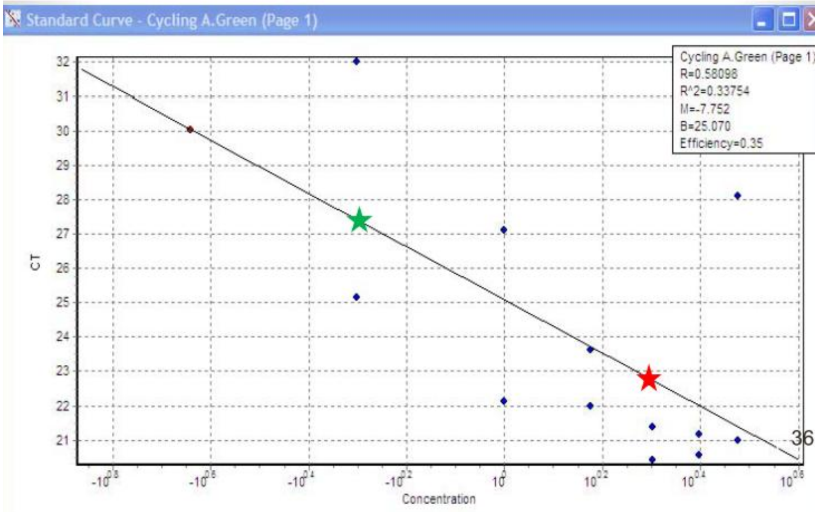
- For greater stability primers should have GC content of around 30-80%
- SYBR green is a non-specific intercalating compound that fluoresces brightly when bound to dsDNA
- Fluorescent reporter probes detect only the DNA containing the sequence complementary to the probe; therefore, use of the reporter probe significantly increases specificity, and enables performing the technique even in the presence of other dsDNA
- It is important to ensure that emission equipment is designed so that it can detect the wavelengths emitted by your probes
- Multiplex qPCR is a method in which multiple targets are amplified in a single reaction. Each target is amplified by a different set of primers, with a uniquely-labeled probe for each



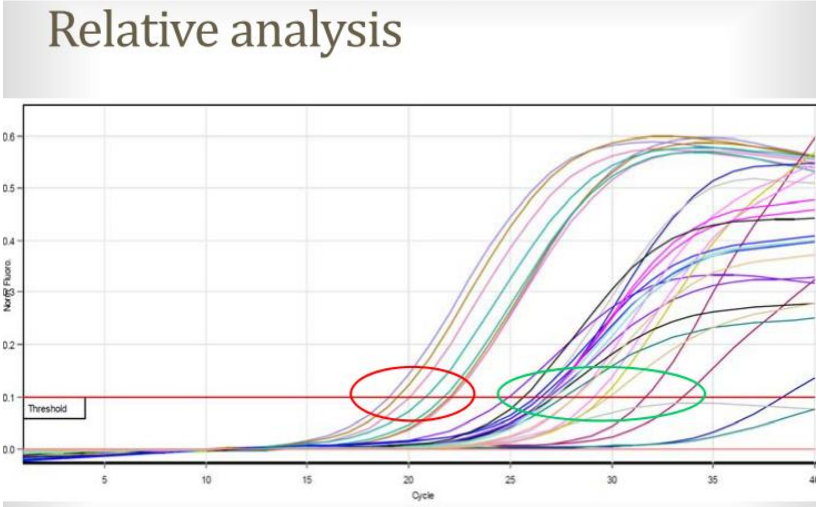
- Probe
  - Expensive
  - Sequence specific
  - Multiplex
  - Sticks on 1 region
  - Detects DNA template extension
- SYBR green
  - Economical
  - Partial sequence specific
  - Singleplex
  - Sticks on every 4 bp
  - Detects double stranded DNA formation

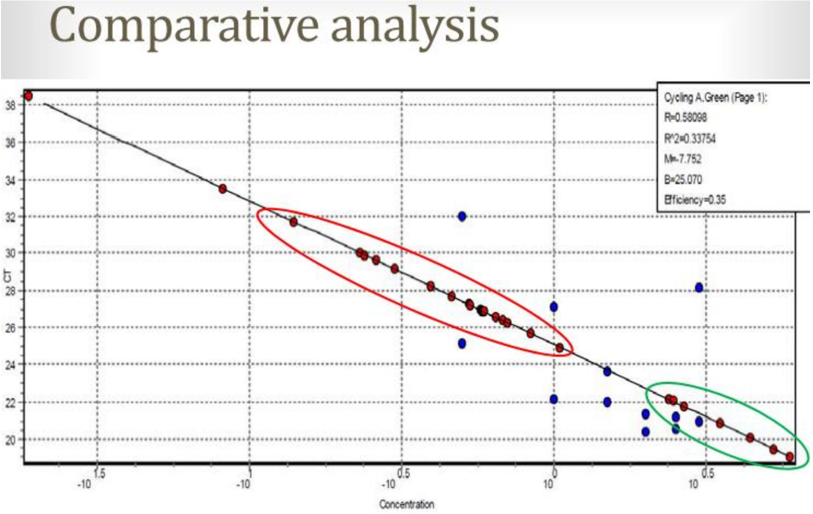
Types of analyses

- Absolute analysis: In absolute quantification using the standard curve method, you quantitate unknowns based on a known quantity. First you create a standard curve; then you compare unknowns to the standard curve and extrapolate a value.
- Relative analysis: In relative quantification, you analyze changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample).
- Comparative analysis: combines both methods


Standard Curve - Cycling A.Green (Page 1)

Cycling A.Green (Page 1)
R=0.58098
R^2=0.33754
M=-7.752
B=25.070
Efficiency=0.35


Relative analysis


Comparative analysis

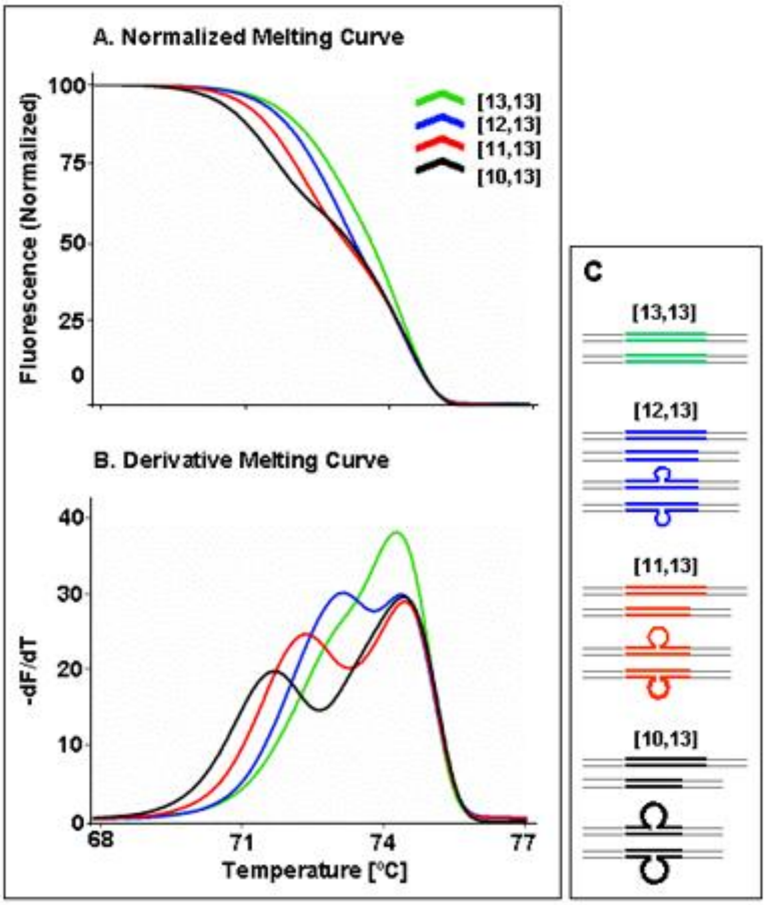
Cycling A.Green (Page 1):
R=0.58098
R^2=0.33754
M=-7.752
B=25.070
Efficiency=0.35

Calculating efficiency

- With perfect efficiency, there will be 100% amplification per cycle
- Actual efficiency will always be less than this
- The actual efficiency can be calculated using the Pfaffl equation
- The following equations give the absolute difference in amount between treated and untreated conditions
- Perfect efficiency: $2^{\Delta\Delta C_t} = 2^{\Delta Ct_{\text{untreat}}(\text{GOI}-\text{HK})-\Delta Ct_{\text{treat}}(\text{GOI}-\text{HK})}$
- Real efficiency: $E^{\Delta\Delta C_t} = \frac{{E_{\text{GOI}}}^{\Delta Ct_{\text{untreat}}(\text{GOI}-\text{HK})}}{{E_{\text{HK}}}^{\Delta Ct_{\text{treat}}(\text{GOI}-\text{HK})}}$
- Amplification efficiency can be improved by improving pipetting skills, optimising primer concentration, and optimising the temperature changes over the course of the cycle
- On a Ct/Log(Co) graph, a slope of -3.3 or -3.4 indicates close to 100% efficiency

Melt curves

- Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. As the temperature is raised, the double strand begins to dissociate leading to a rise in the absorbance intensity, hyperchromicity
- The temperature at which 50% of DNA is denatured is known as the melting point, though it is an inaccurate term as it has very little to do with a traditional melting point
- The graph of the negative first derivative of the melting-curve may make it easier to pin-point the temperature of dissociation (defined as 50% dissociation), by virtue of the peaks thus formed



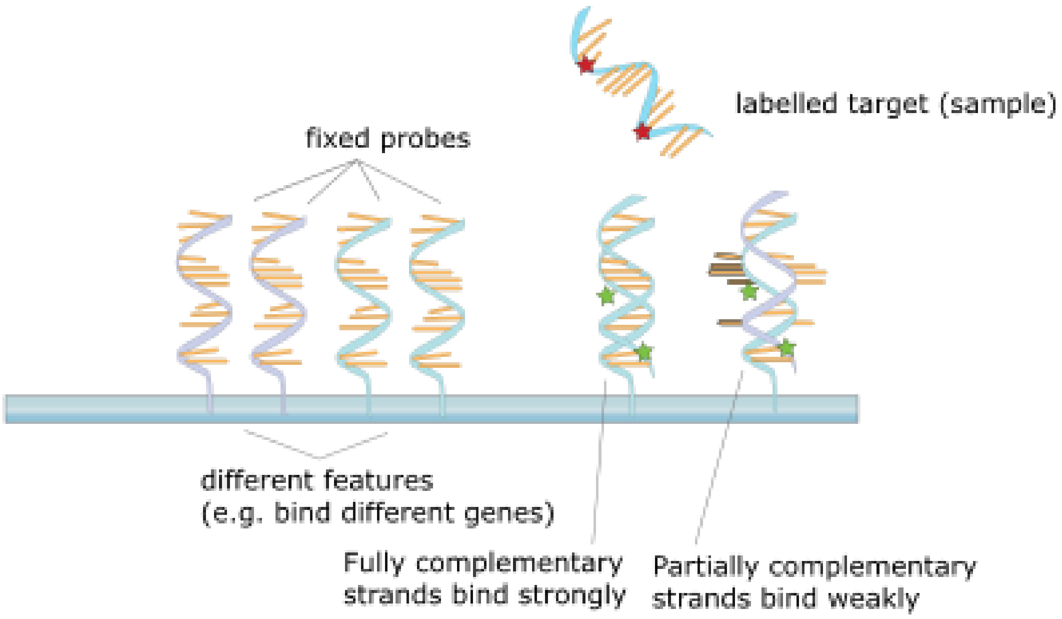
Transcriptomics

The transcriptome

- The transcriptome is the set of all RNA molecules including mRNA, rRNA and tRNA, that are transcribed in a cell or population of cells
- It reflects the genes that are being actively expressed at any given time
- Each gene may produce more than one variant of mRNA because of alternative splicing, RNA editing, or alternative transcription initiation and termination sites
- According to 2012 ENCODE, ~75% of noncoding DNA in the human genome did undergo active transcription

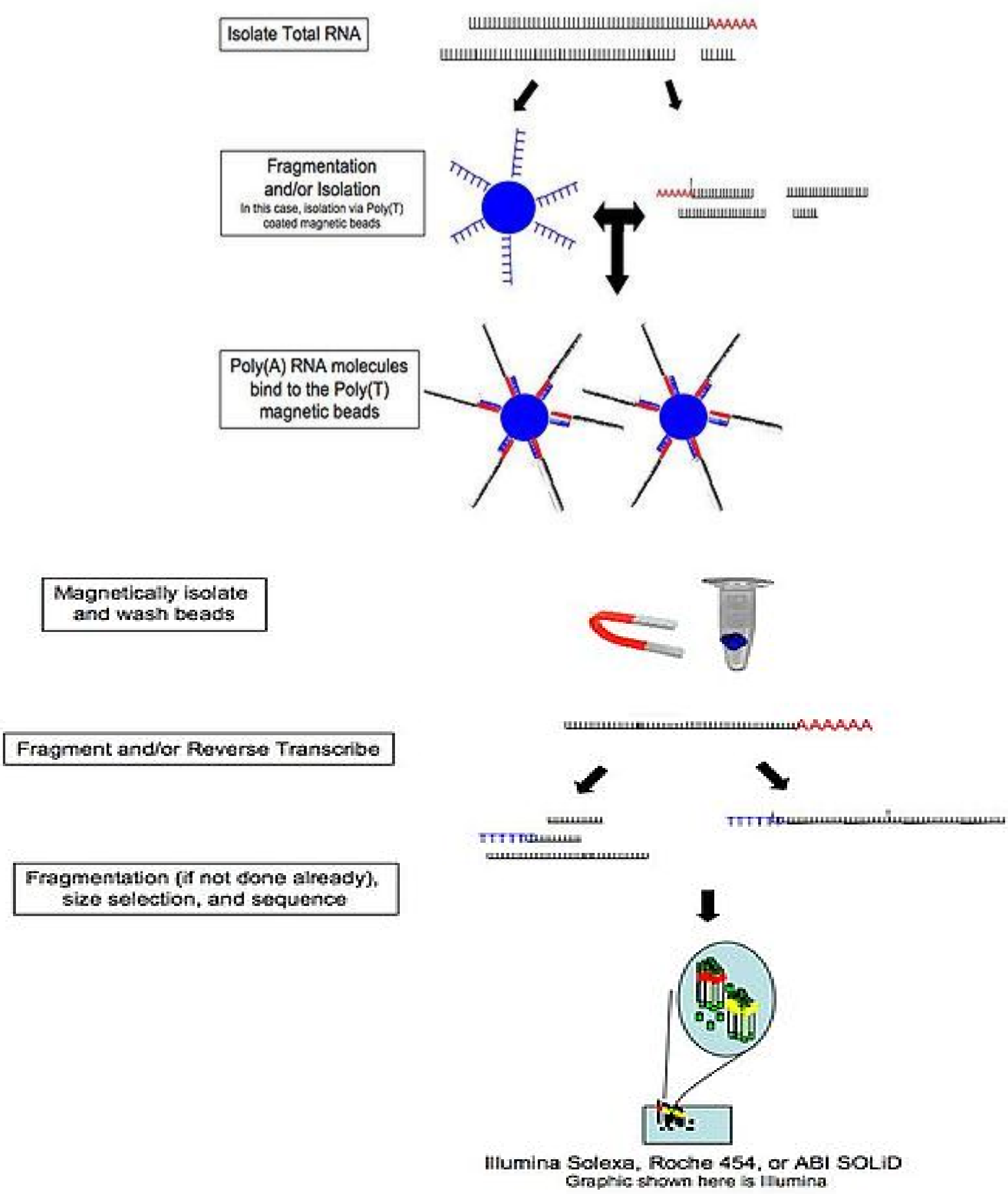
DNA microarrays

- A DNA microarray (chip or biochip) is a collection of microscopic DNA spots (probes) attached to a solid surface
- Probes can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample (called a target)

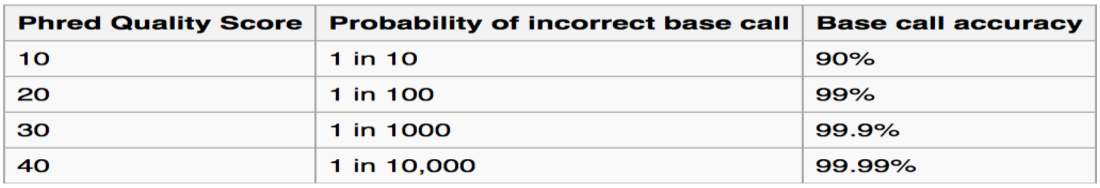


RNA-seq

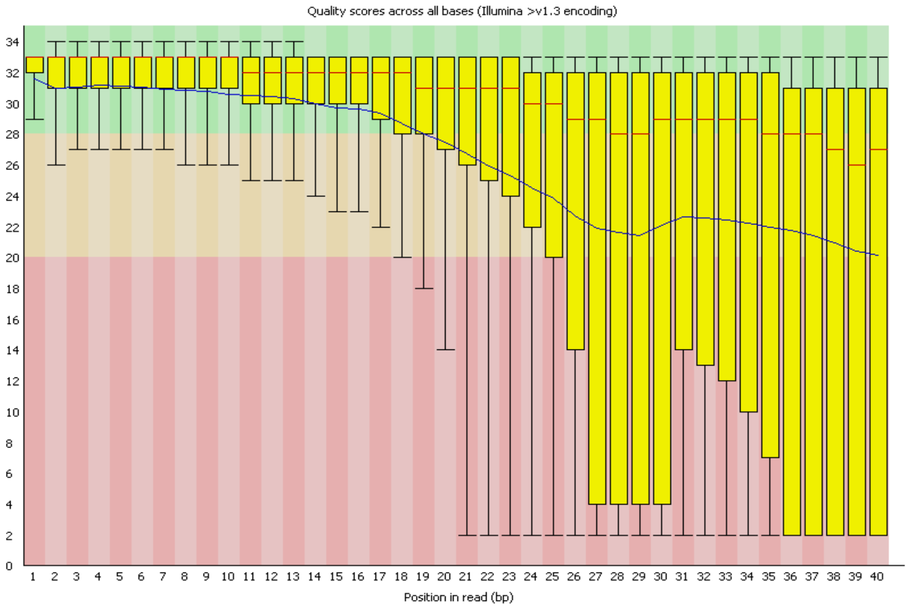
- RNA-seq, or whole transcriptome shotgun sequencing, is a technique for using next-gen sequencing to identify all RNA in a sample
- It is a superior technique to microarrays because it does not require transcript-specific probes or knowledge of the sequences
- RNA-seq also is not limited by the fluorescence background and saturation low and high end limits that plague microarrays, instead providing digital sequencing read counts across a wider dynamic range of magnitudes. This also means it can detect very rare transcripts

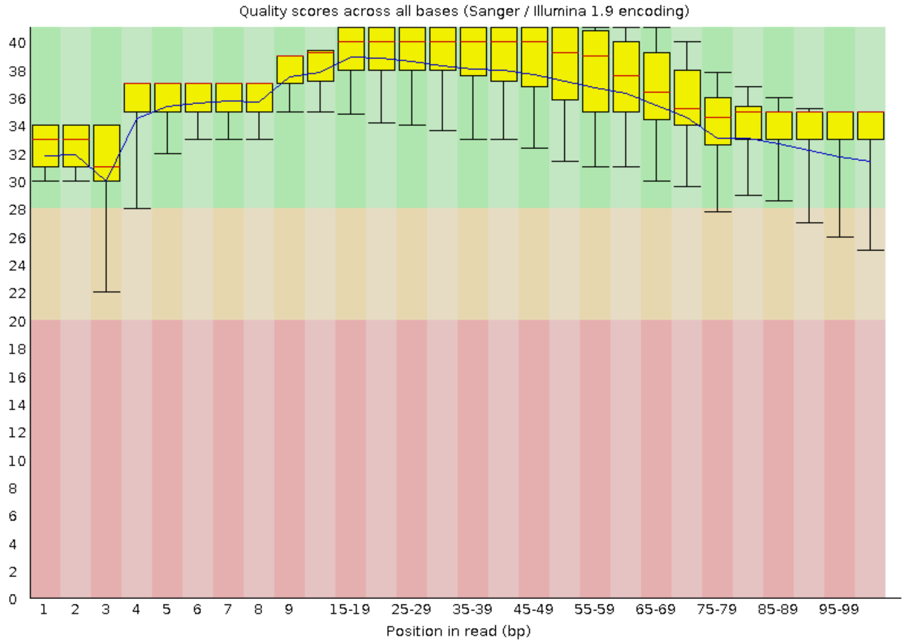

Isolate Total RNA
AAAAAA
Fragmentation and/or Isolation
In this case, isolation via Poly(T) coated magnetic beads
Poly(A) RNA molecules bind to the Poly(T) magnetic beads
Magnetically isolate and wash beads
Fragment and/or Reverse Transcribe
AAAAAA
TTTTT
Fragmentation (if not done already), size selection, and sequence
Illumina Solexa, Roche 454, or ABI SOLiD
Graphic shown here is Illumina

- All reads are assigned a quality Q score between 0 and 40, where $Q = -10\log_{10} P$ for P the probability of an incorrect base assignment
- One useful technique is to plot the boxplot results for the quality scores across all reads by the position in the read
- This allows us to compare the quality of different sequencing methods, for instance 2009 Illumina (top) with 2013 Illumina HiSeq2000 (bottom)
- A cutoff for Q=20 is typically used for discarding low quality data
- After quality B-trimming any trimming artifacts need to be corrected, such as removing uninformative sequences (N), polyA tails, and contaminants from primers or adapters



Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%





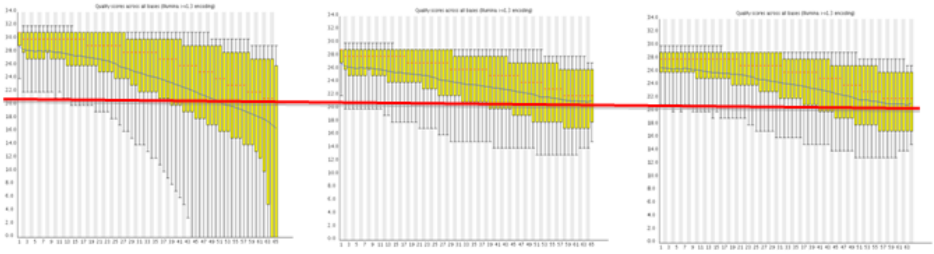
raw → B-trimmed → trimming artifacts

Illumina_s4_Aug09.fastq
Total Sequences 12,505,533,
Sequence length 65
%GC 47

s4_Aug09_B_trimmed.fastq
Total Sequences 12,503,363
Sequence length 10-65
%GC 46

s4TrimmedSno.fastq
Total Sequences 10,412,993
Sequence length 50-64
%GC 45

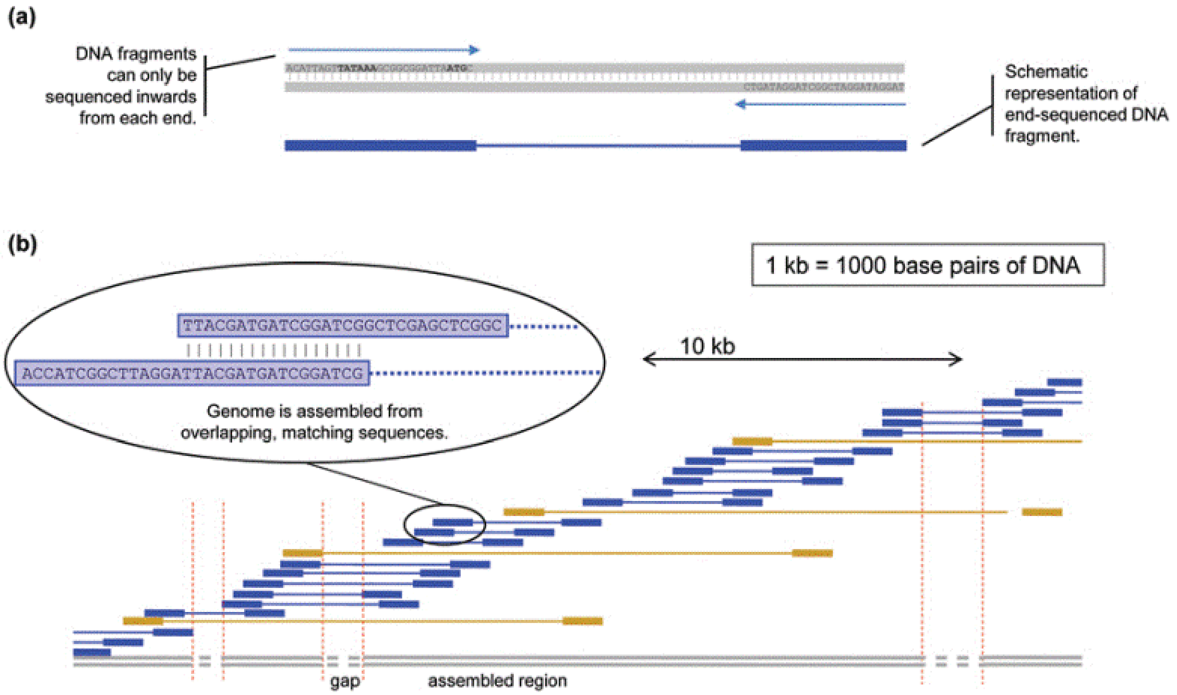
17% removed

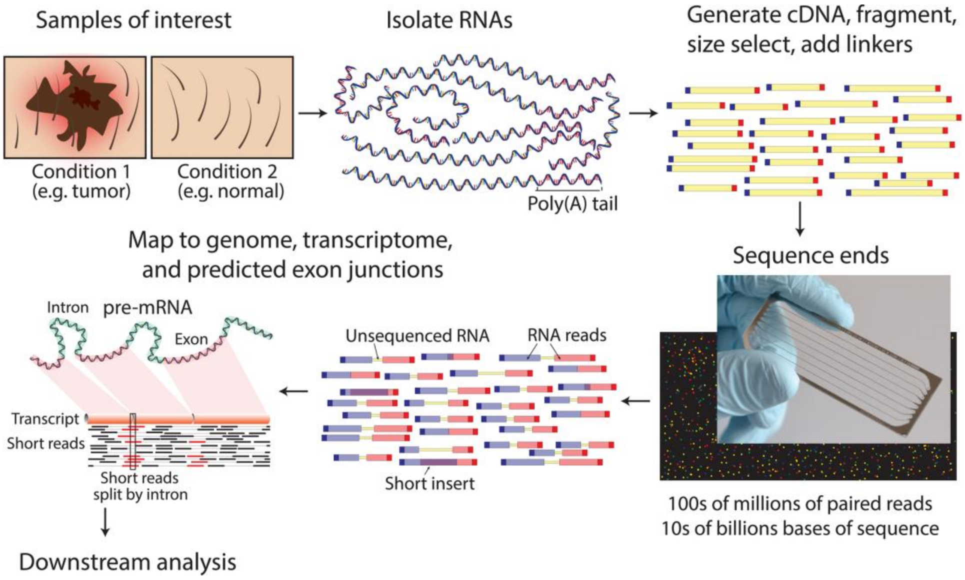


Q=20

De novo sequence assembly

- De novo sequence assembly involves assembling many short reads into contigs
- Often based on multiple K-mer assembly using contigs of length K, varying K to get optimal results
- A small K-mer means more reads can be assembled (more contigs), but the contigs are shorter
- In this method N50 refers to the median contig size

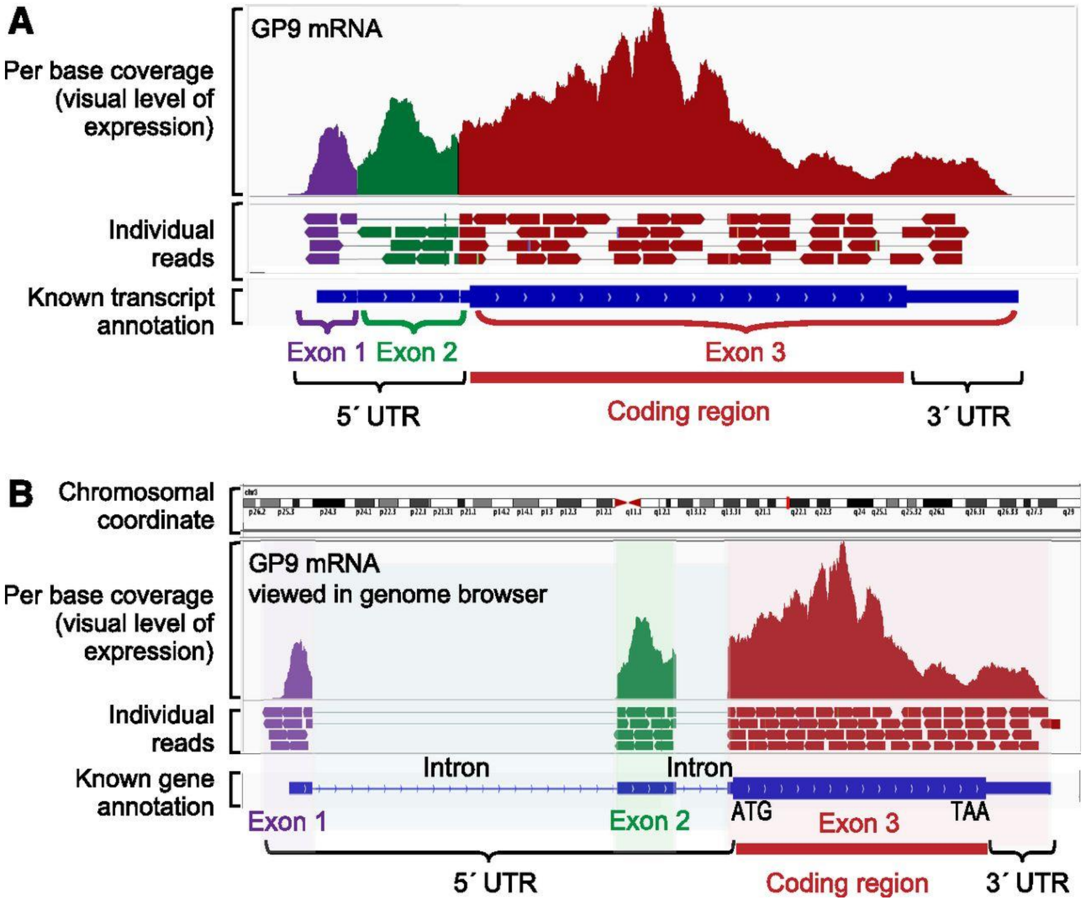

(a)
DNA fragments can only be sequenced inwards from each end.
Schematic representation of end-sequenced DNA fragment.
(b)
1 kb = 1000 base pairs of DNA
TTACGATGATCGGATCGGCTCGAGCTCGGC
ACCATCGGCTTAGGATTACGATGATCGGATCG
Genome is assembled from overlapping, matching sequences.
10 kb
gap
assembled region


Samples of interest
Condition 1 (e.g. tumor)
Condition 2 (e.g. normal)
Isolate RNAs
Poly(A) tail
Generate cDNA, fragment, size select, add linkers
Sequence ends
100s of millions of paired reads
10s of billions bases of sequence
Unsequenced RNA
RNA reads
Short insert
Map to genome, transcriptome, and predicted exon junctions
Intron
pre-mRNA
Exon
Transcript
Short reads
Short reads split by intron
Downstream analysis

Differential gene expression

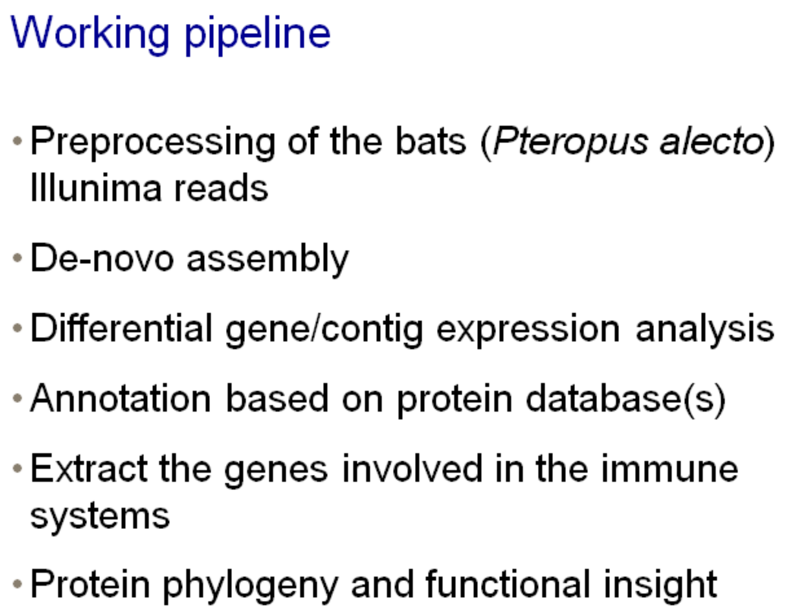
- Following sequencing, diferential gene expression analysis involves aligning the contigs to a genome to work out the position of introns and coding regions

- To identify biological significant differentially expressed genes, need to first normalise for total number of read differences
- Annotation is based on hits from blastx searches against known proteins, with reference to (for example) nr database, which is a non-redundant protein database



Example bat immune study

- One study examined the expression of immune-related renes in the *Pteropus alecto* (black flying fox bat) to better understand their role in acting as disease reservoirs for humans and domestic animals
- As of 2010 only two poor quality bat genomes had been sequenced, thus posing a challenge for de-novo genome assembly
- Tissue was taken from the thymus and spleen of two bats, and was then sequenced and subject to to de-novo assembly and differential gene expression analysis, focusing on immune system genes
- After contig assembly, about 45% of the contigs of length 21-31 were able to be identified with known proteins, which were found to come from a very wide range of species
- Preliminary annotation of the bat transcripts have revealed many interesting immune genes, such as MHC class I, TLRs and interferon related genes



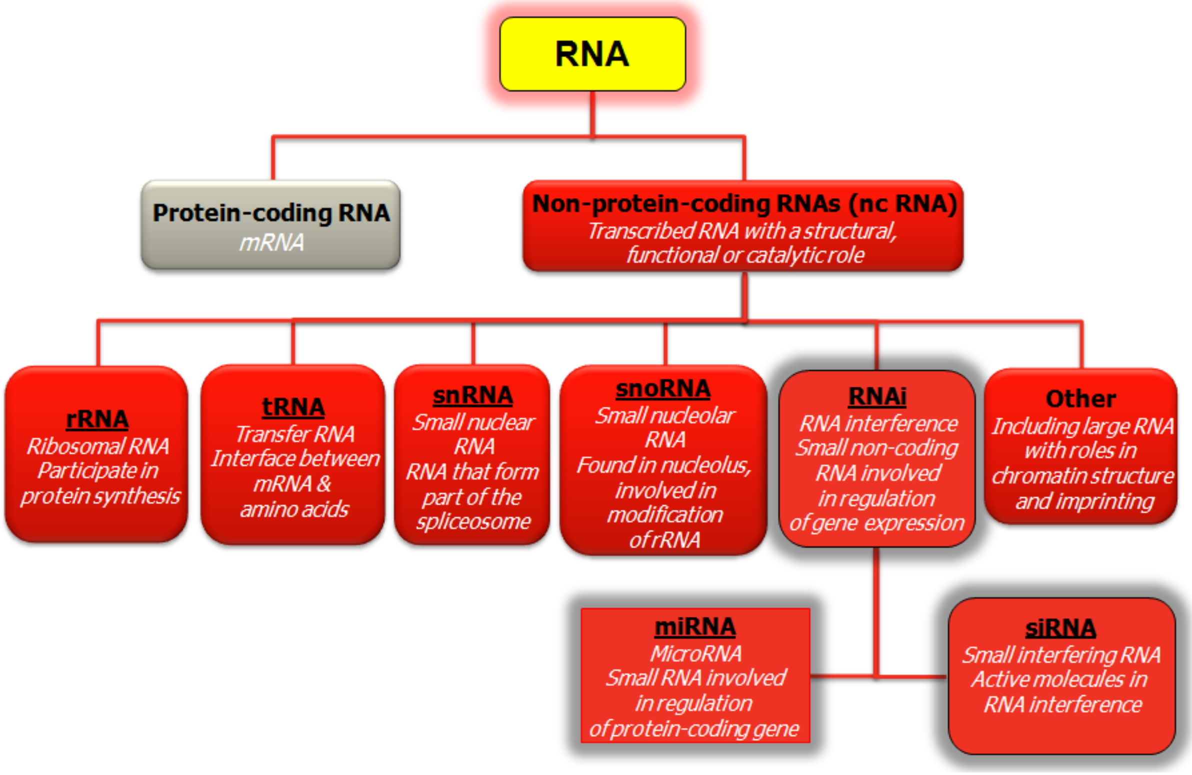
Working pipeline

- Preprocessing of the bats (*Pteropus alecto*) Illunima reads
- De-novo assembly
- Differential gene/contig expression analysis
- Annotation based on protein database(s)
- Extract the genes involved in the immune systems
- Protein phylogeny and functional insight

microRNA

Non-coding RNA

- Most mammals and other vertebrates have approximately same number of genes: 20-25k in animas, 27-40k in plants
- The number of protein coding sequences is thus conserved (orthologous) in primitive to advanced animals. What is it, then, that makes humans to different?
- One part of the answer seems to be that humans have a much higher proportion of non-coding RNA than most other animals
- There are many different types of RNA found in any given cell



RNA

Protein-coding RNA
mRNA

Non-protein-coding RNAs (nc RNA)
Transcribed RNA with a structural, functional or catalytic role

rRNA
Ribosomal RNA Participate in protein synthesis

tRNA
Transfer RNA Interface between mRNA & amino acids

snRNA
Small nuclear RNA RNA that form part of the spliceosome

snoRNA
Small nucleolar RNA Found in nucleolus, involved in modification of rRNA

RNAi
RNA interference Small non-coding RNA involved in regulation of gene expression

Other
Including large RNA with roles in chromatin structure and imprinting

miRNA
MicroRNA Small RNA involved in regulation of protein-coding gene

siRNA
Small interfering RNA Active molecules in RNA interference

Discovery of miRNA

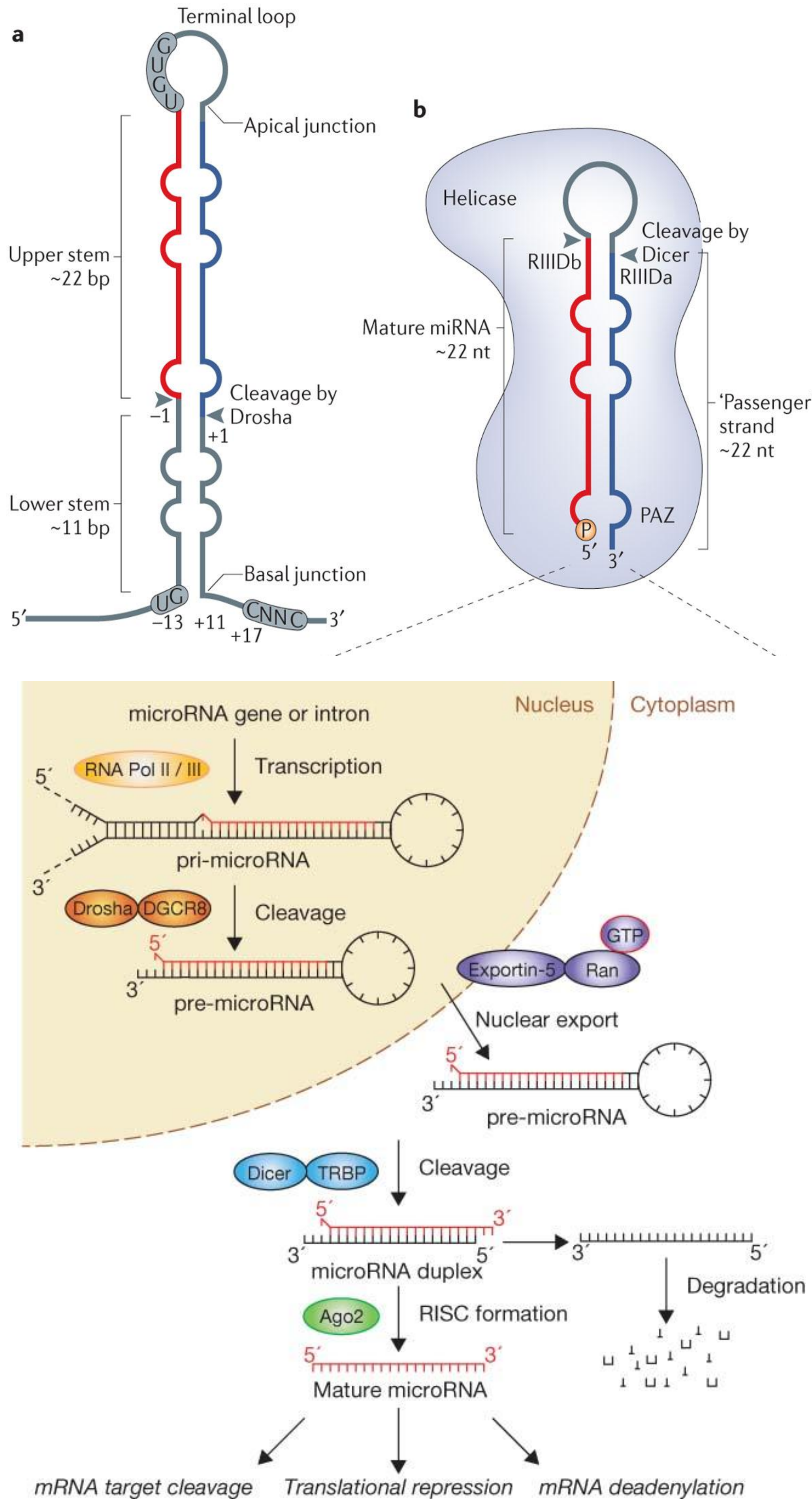
- miRNA was discovered in 1993 when Victor Ambros and colleagues discovered that lin-4, a gene known to control the timing of C. elegans larval development, does not code for a protein but instead produces a pair of small RNAs ~22 nt, and ~61 nt in length
- Seven years later, another non-coding gene let7 was found to regulate worm development, and has since been found to be conserved in number of species including humans
- Since 2000, thousands of miRNAs have been reported from a number of species including humans
- miRNA are now named sequentially with the prefix mir, so mir-125a. The same miRNA generated from different loci are denoted with a final latter, so mir-125b-2

Transcriptional regulation of miRNAs

- The majority of canonical miRNAs are encoded by introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions
- miRNAs in the same cluster are generally co-transcribed, but the individual miRNAs can be additionally regulated at the post-transcriptional level
- Some miRNA genes reside in the introns of protein-coding genes and, thus, share the promoter of the host gene
- miRNA genes often have multiple transcription start sites
- miRNA transcription is carried out by RNA Pol II
- Transcription factors include p53, MYC, ZEB1 and ZEB2, and MYOD1

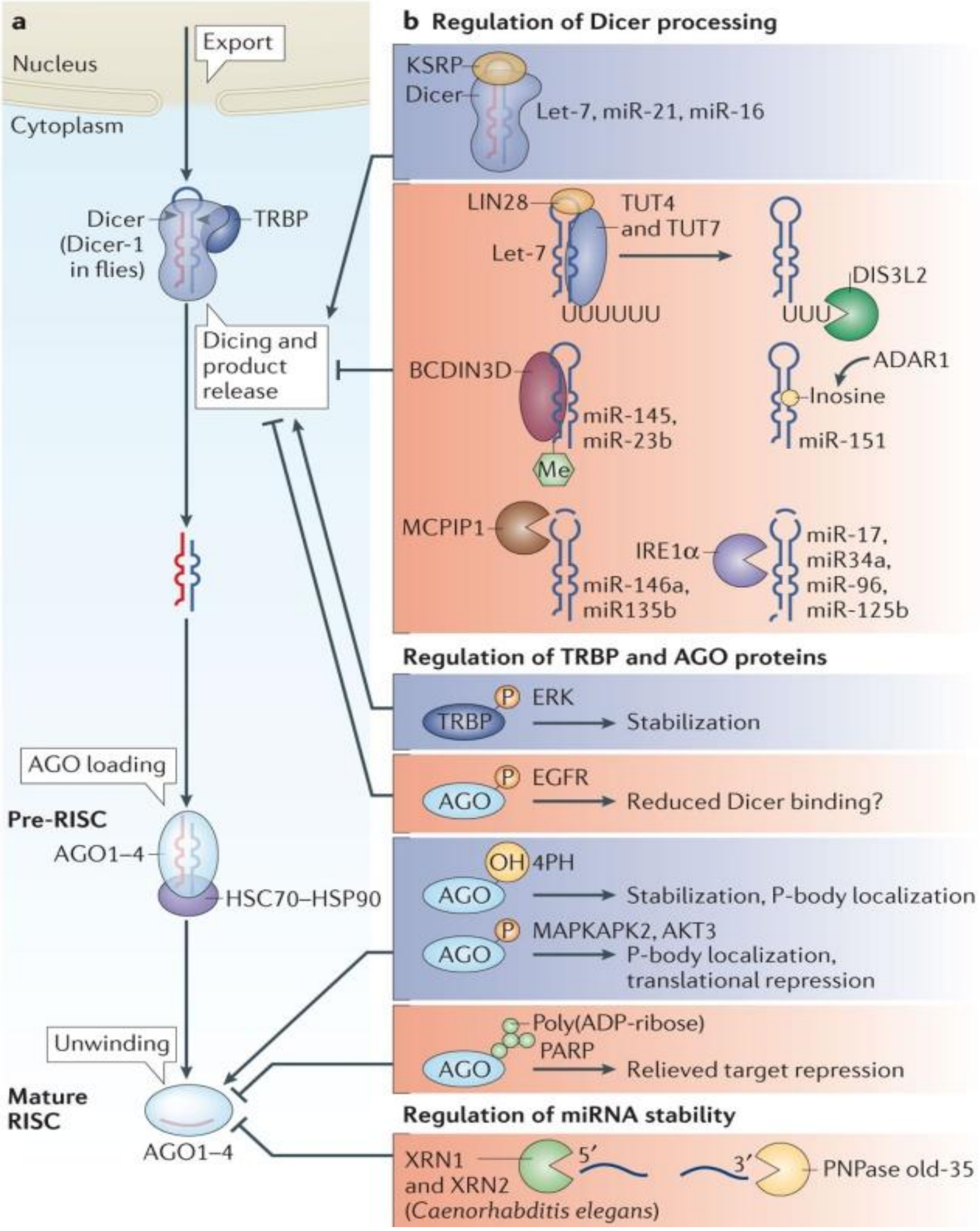
Nuclear processing of miRNAs

- Following transcription, the primary miRNA (pri-miRNA) undergoes several steps of maturation
- pri-miRNA is long (typically over 1 kb) and contains a local stem–loop structure, in which mature miRNA sequences are embedded
- The nuclear RNase III Drosha initiates the maturation process by cropping the stem–loop to release a small hairpin-shaped RNA of ~65 nucleotides in length (pre-miRNA).
- Together with its essential cofactor DGCR8, Drosha forms a complex called Microprocessor
- As Drosha cleavage defines the terminus of an miRNA and thereby determines its specificity, it is important that Microprocessor precisely recognizes and cleaves a pri-miRNA
- Drosha cleaves the hairpin at approximately 11 bp away from the 'basal' junction, which thus functions as the major reference point in determining the cleavage site


a
Terminal loop
G
U
G
U
Apical junction
Upper stem
~22 bp
Cleavage by
Drosha
−1
+1
Lower stem
~11 bp
Basal junction
5′
UG
−13
+11
CNNC
+17
3′
b
Helicase
Cleavage by
Dicer
RIIIDb
RIIIDa
Mature miRNA
~22 nt
'Passenger'
strand
~22 nt
PAZ
P
5′
3′
Nucleus
Cytoplasm
microRNA gene or intron
RNA Pol II / III
Transcription
5′
3′
pri-microRNA
Drosha
DGCR8
Cleavage
5′
3′
pre-microRNA
Exportin-5
Ran
GTP
Nuclear export
5′
3′
pre-microRNA
Dicer
TRBP
Cleavage
5′
3′
3′
5′
microRNA duplex
3′
5′
Degradation
Ago2
RISC formation
5′
3′
Mature microRNA
mRNA target cleavage
Translational repression
mRNA deadenylation

Cytosolic processing of miRNAs

- Dicer interacts with a double-stranded RNA-binding domain (dsRBD) protein-binding protein (TRBP)
- Following Dicer processing, the RNA duplex is released and subsequently loaded onto human Argonaute (Ago)
- A heat shock cognate 70 (HSC70)–heat shock protein 90 (HSP90) complex hydrolyses ATP to load the RNA duplex
- The 'passenger' strand is discarded and the mature microRNA (miRNA) remains in one of the AGO proteins forming a RISC (RNA-induced silencing complex)

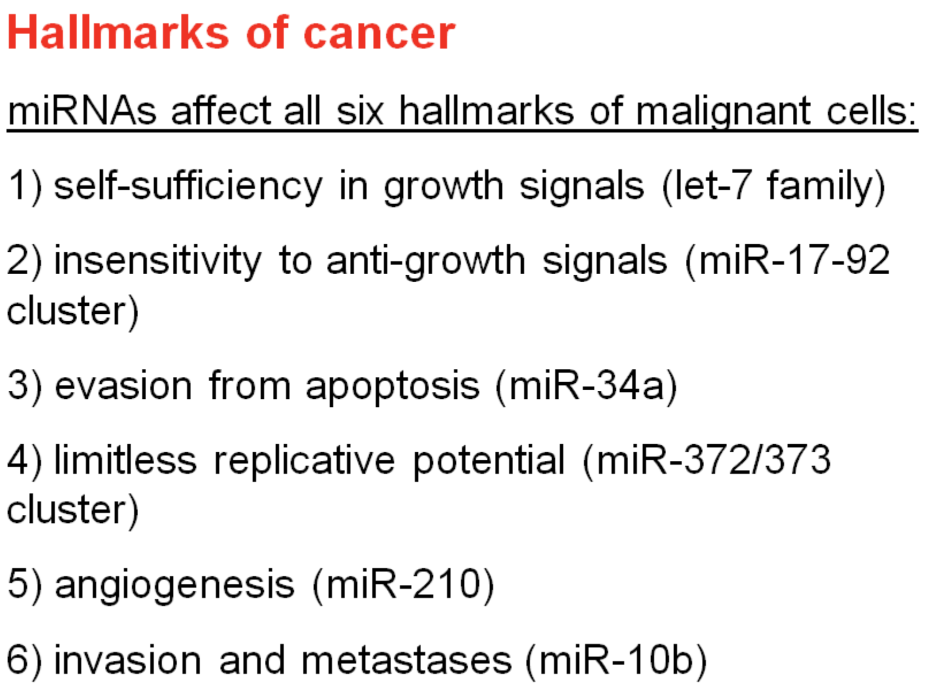


miRNA in inflammation

- miRNAs have recently been implicated in the inflammatory response, their transcription controlled by protein regulators
- p53, KSRP, and Smad are proteins that interact with miRNA loops and are activated during inflammatory responses
- Inflammatory cytokines, such as interferons (IFNs), can repress expression of biogenesis factors including Dicer

miRNA in cancer

- Tumorogenesis involves genes associated with inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and invasion. miRNA can potentially regulate expression of most these genes
- Croce et al. showed that miR-15a/16-1 cluster is frequently deleted in chronic lymphocytic leukemia, implicating these miRNAs as tumor suppressors
- All the tumors analyzed have a specific miRNA signature, “miRNome”, that characterizes the malignant state and defines some of the clinico-pathological features of the tumors (grade, stage, sex, age, aggressiveness, vascular invasion, proliferation index, even tissue of origin)
- Most miRNAs have been found repressed in cancers relative to normal tissue, indicating a repressive role
- Likewise, global depletion of miRNAs by genetic deletion of the miRNA-processing machinery favours cell transformation and tumorogenesis in vivo
- There are, however, several miRNAs that are up-regulated in tumor cells, indicating an oncogenic role
- Two particularly interesting results:
  - transgenic expression of miR-155 or miR-21 and deletion of miR-15a/16-1 are sufficient to initiate lymphomagenesis in mice
  - systemic delivery of selected miRNAs let-7, miR-26a, miR-34a, and miR-143/145 inhibits tumor progression *in vivo*



Hallmarks of cancer

miRNAs affect all six hallmarks of malignant cells:

1) self-sufficiency in growth signals (let-7 family)

2) insensitivity to anti-growth signals (miR-17-92 cluster)

3) evasion from apoptosis (miR-34a)

4) limitless replicative potential (miR-372/373 cluster)

5) angiogenesis (miR-210)

6) invasion and metastases (miR-10b)

TGA website submission

Primary orality lynne kelly